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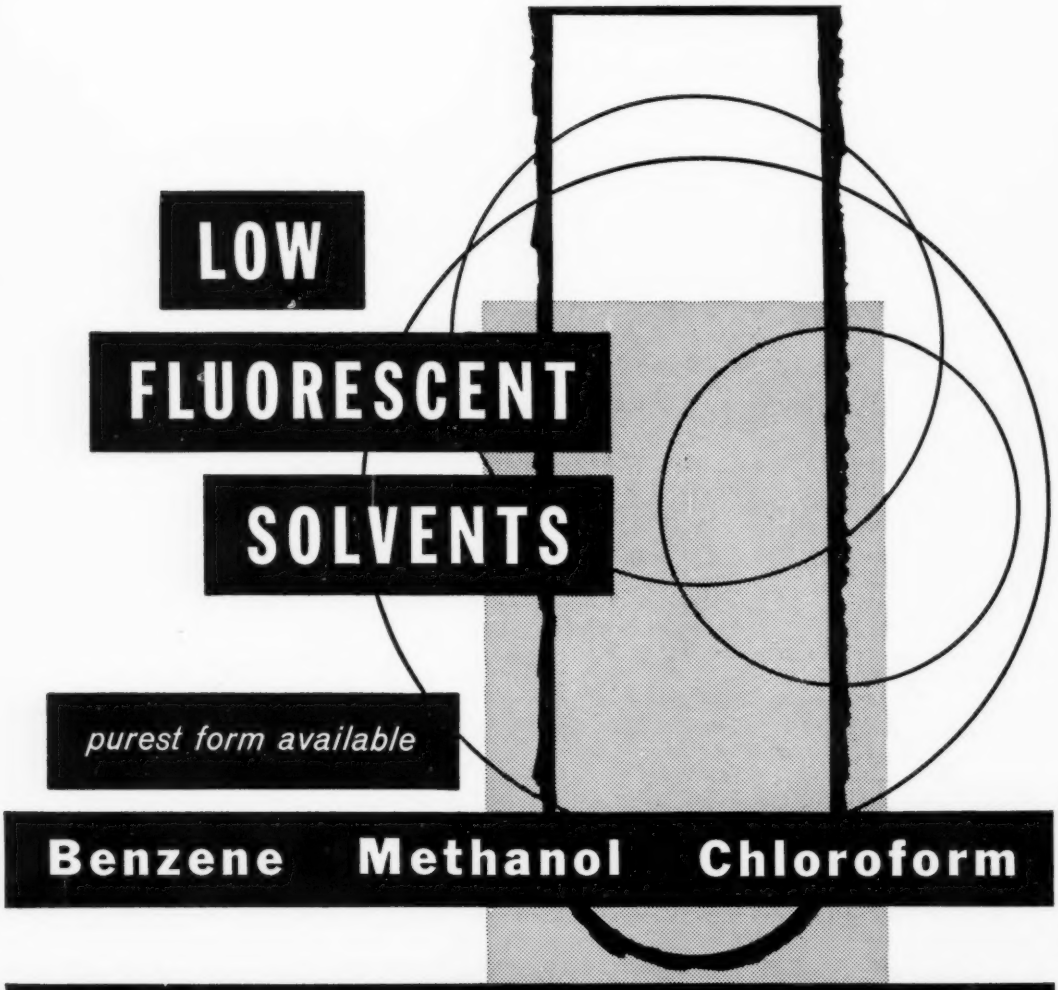

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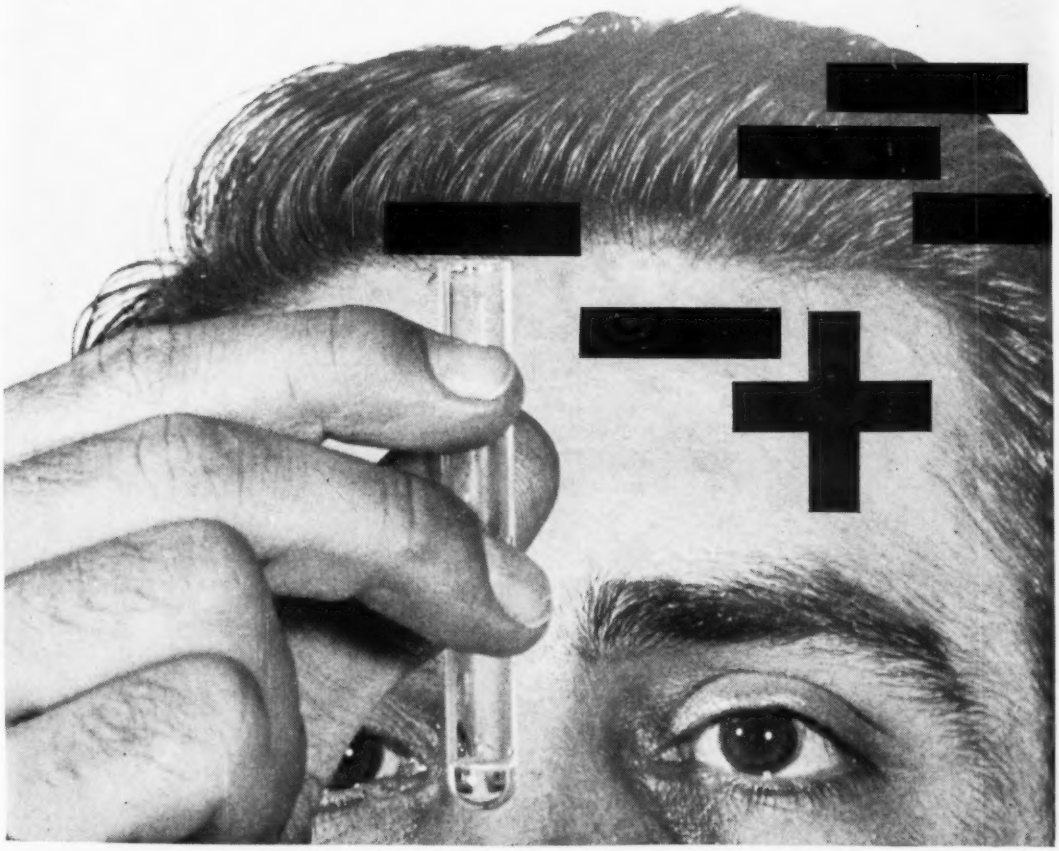

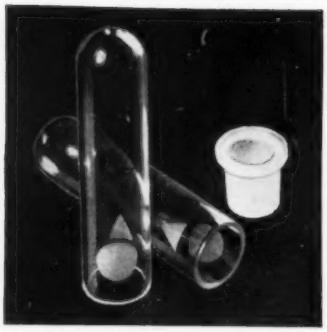

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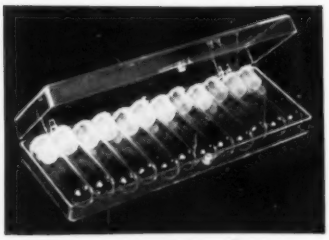

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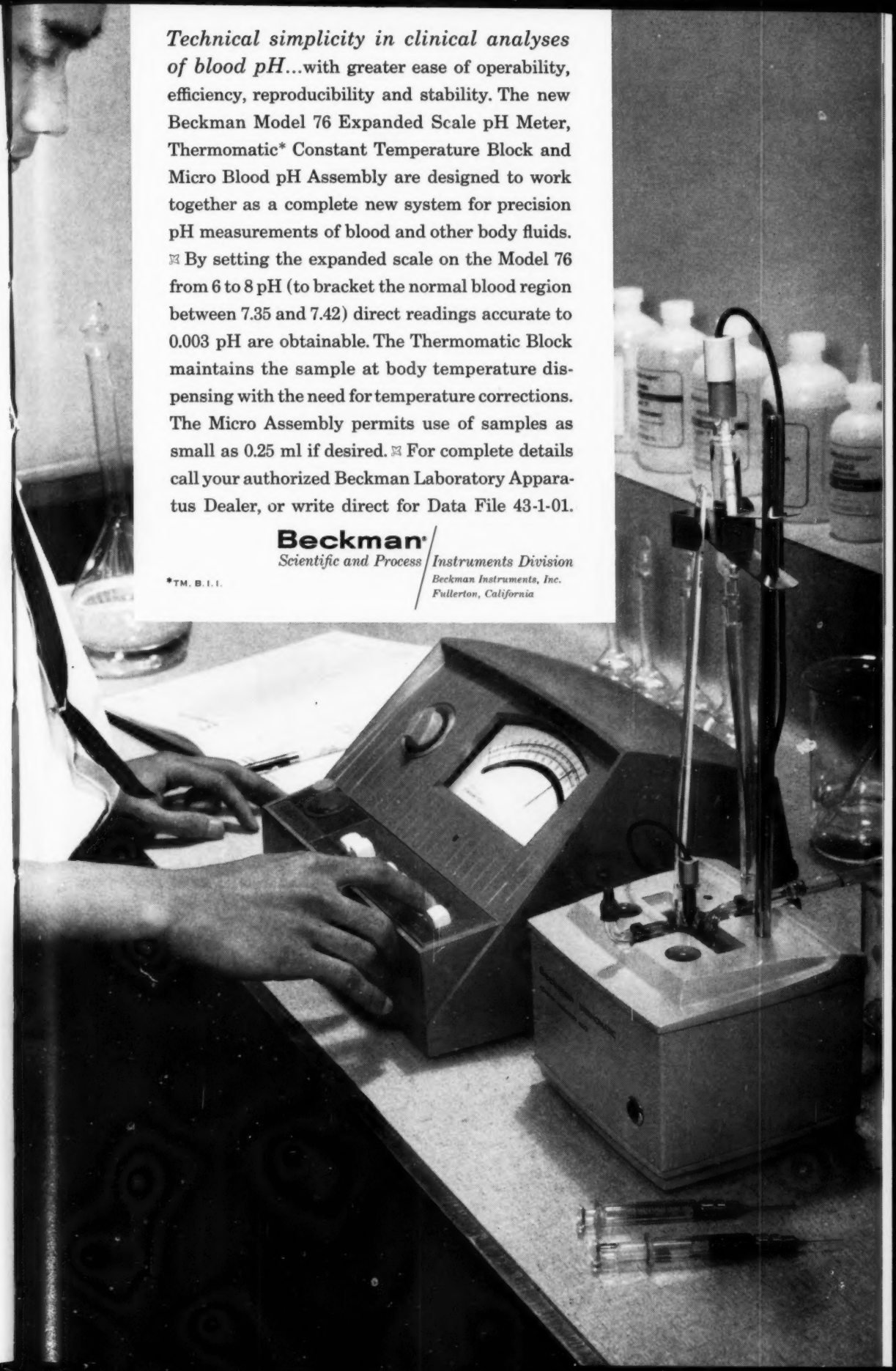

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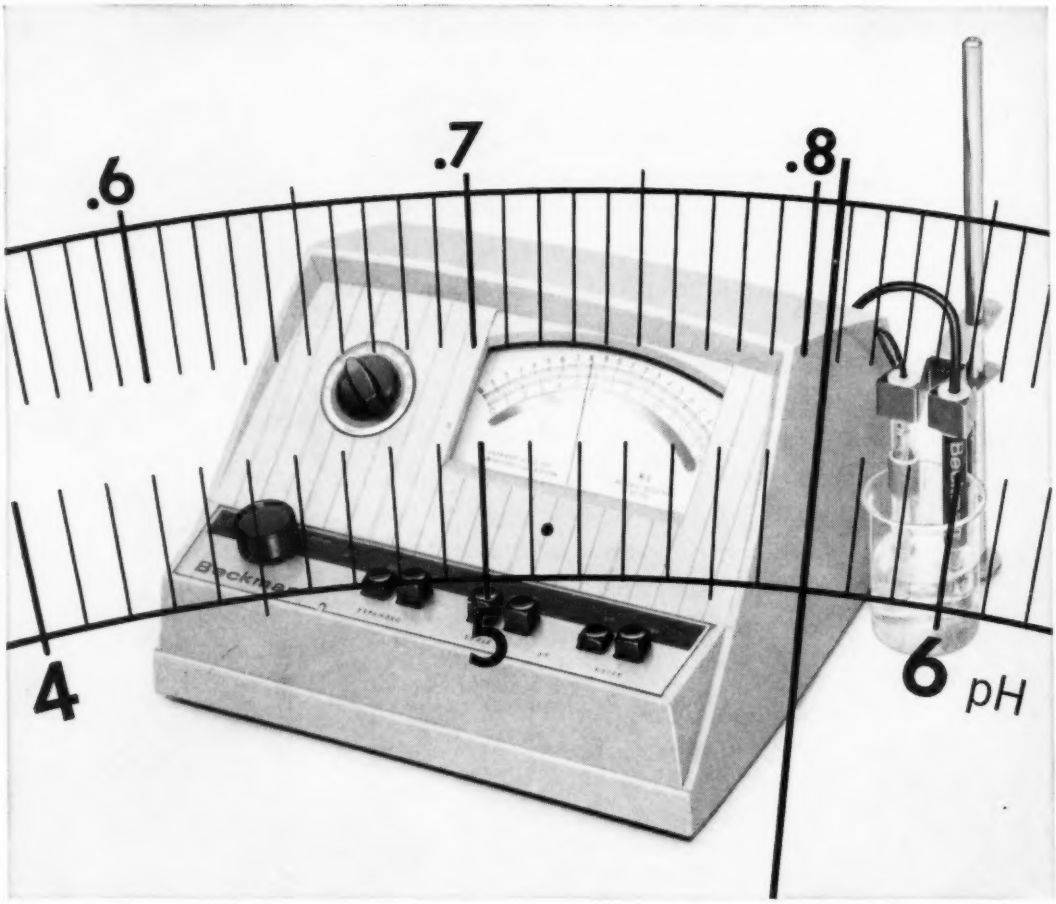

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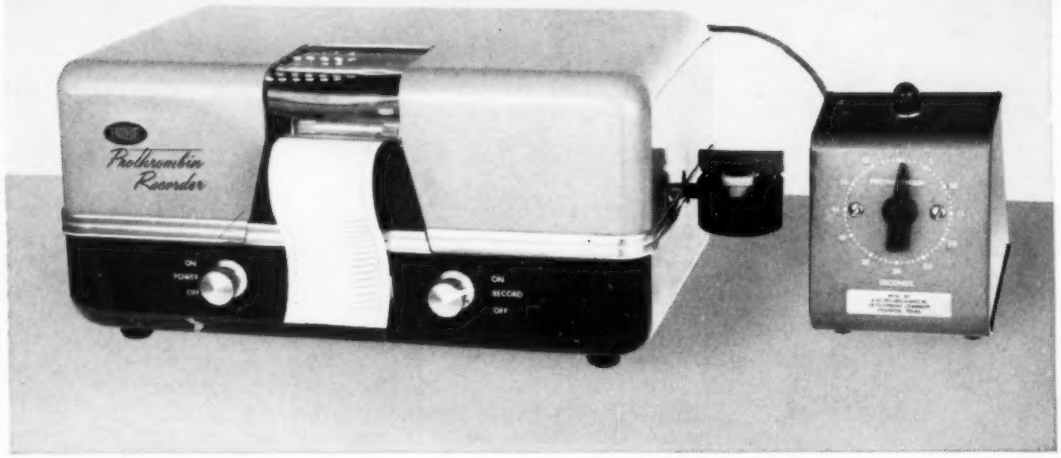

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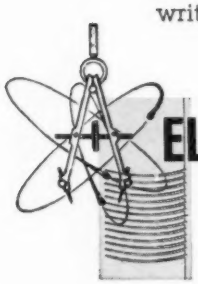

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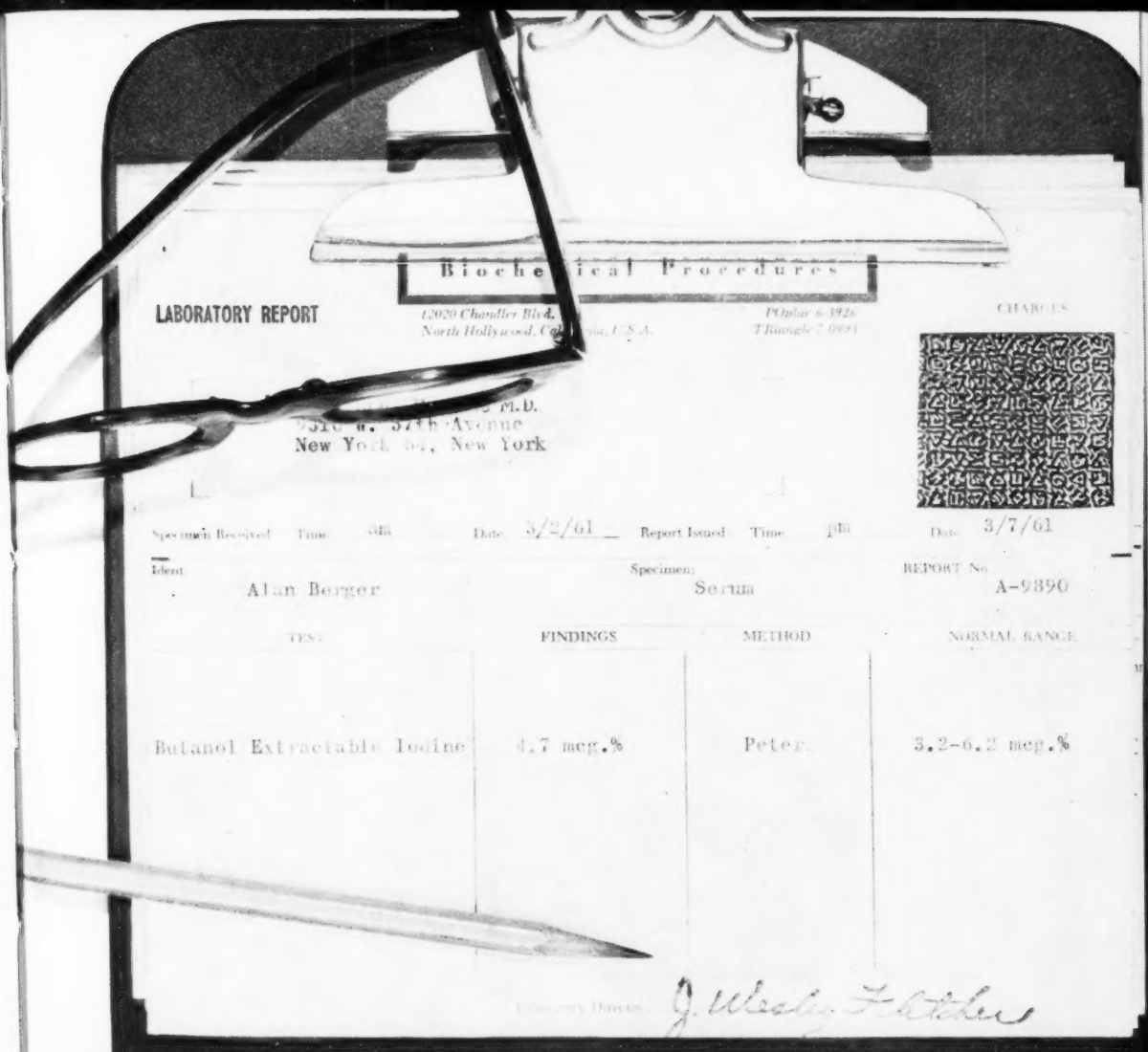

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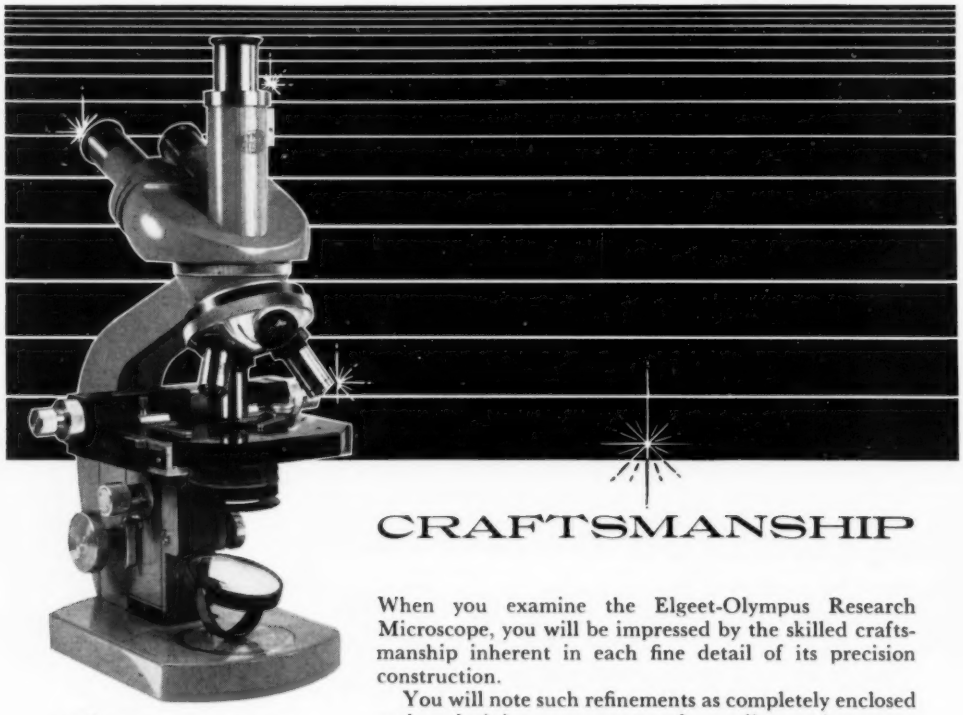

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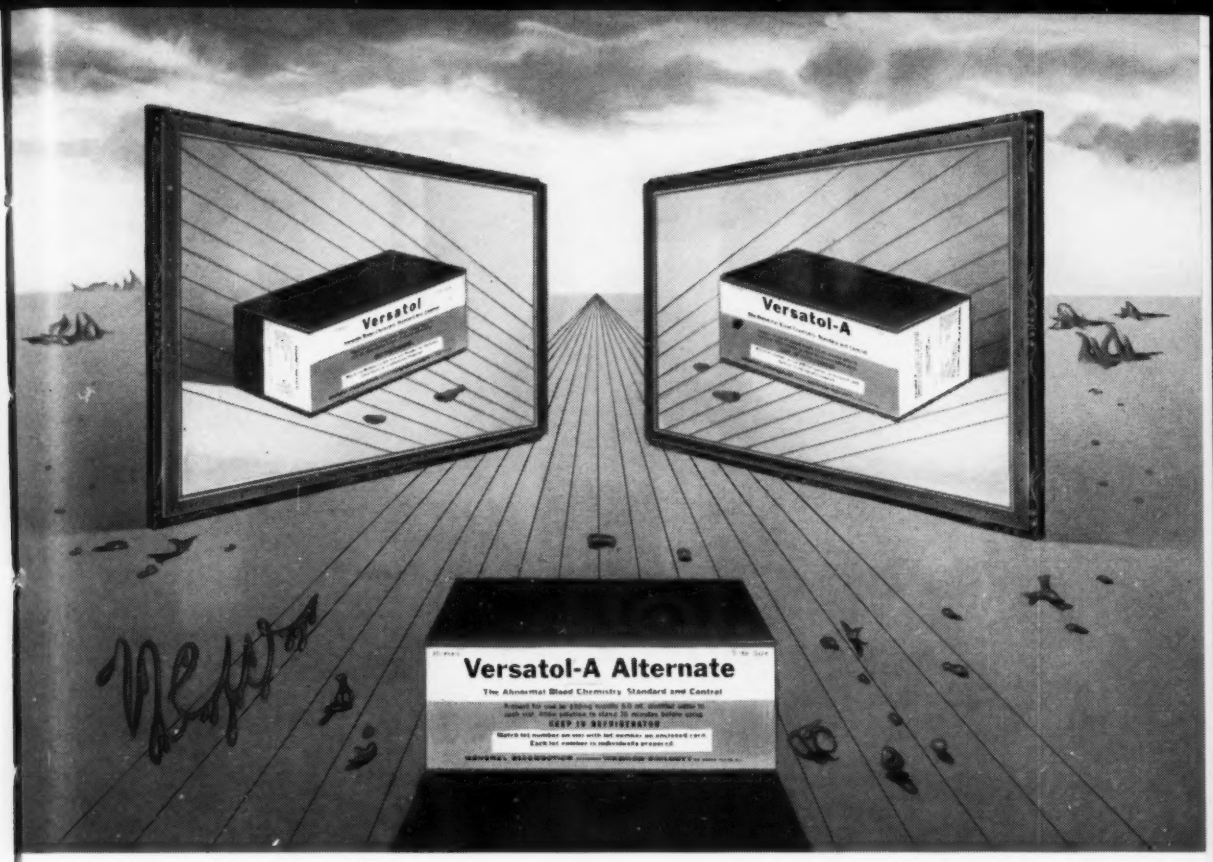

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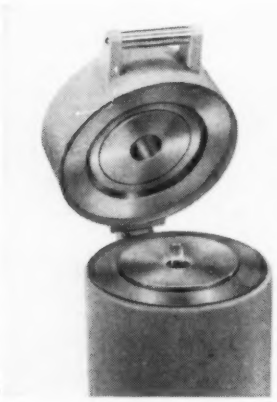


1.

2.

1. Counterbalanced lid raises easily. Small central plug in lid can be removed if test tubes longer than 150 mm are used. Plug can be left in place and lid lowered on tubes up to 150 mm long. Splash guard protects crystal and shielding from radioactive spills.

2. Top view. Lead ring is removed to expose top of crystal. Bottles up to 5½" in diameter can be positioned directly on top of the crystal (see figure 3).

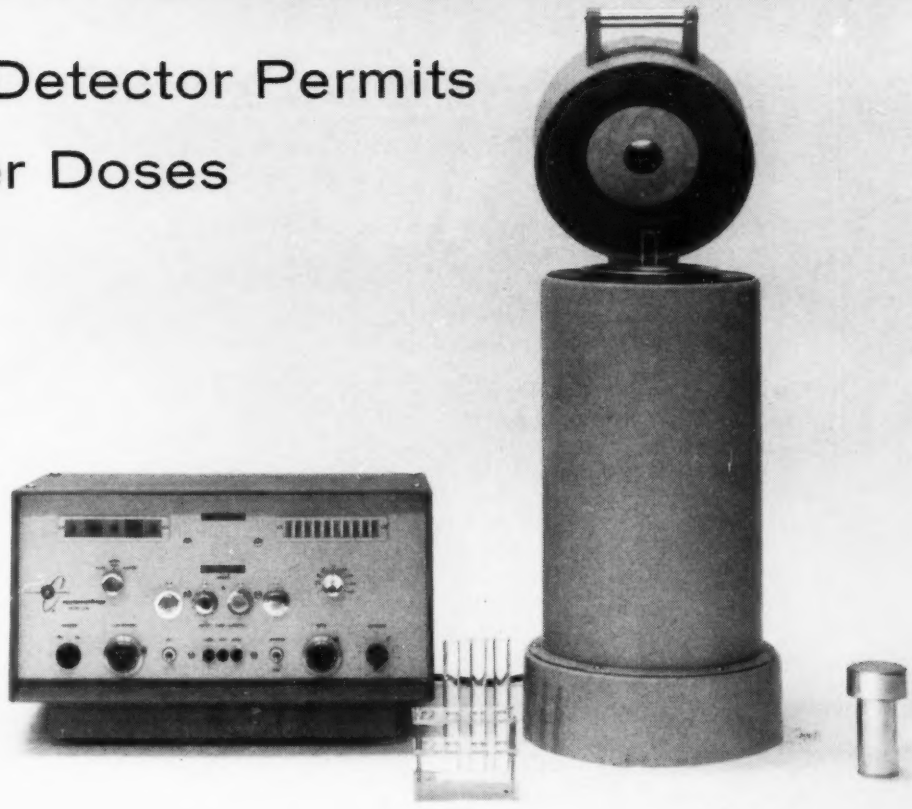
3.

4.

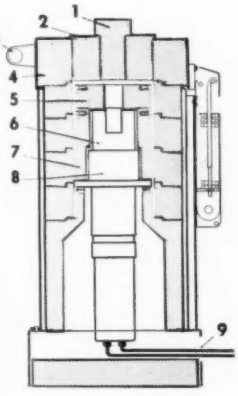
3. Bottle positioned in shield. Large plug in lid has been removed so that cover can be closed around bottle.

4. Lead ring surrounding the crystal can also be removed so that re-entrant type containers can be placed around the crystal.

Well Detector Permits Tracer Doses



Nuclear-Chicago DS-303 Scintillation Well Detector is ideal for use with Model 132A Analyzer Computer which combines a scaler and gamma-ray spectrometer in one chassis.



Cross-section of DS-303 Well Detector

1. Removable Plug
2. Removable Plug
3. Handle
4. Counterbalanced Lid
5. Removable Ring
6. Crystal
7. Removable Ring
8. Scintillation-Probe
9. Cables to Scaler, Spectrometer, or Ratemeter.

Extensive Shielding Minimizes Background. The DS-303 provides more shielding than any other commercial scintillation detector. The 3" crystal is completely surrounded—at the top, bottom and sides—by lead. At the sides and top the lead shielding varies from 3½" to 5½". At the base of the detector, 2" of lead is used. This extensive shielding lowers background to only 350 counts per minute, or less, using a standard scaler of quarter-volt sensitivity. When a gamma-ray spectrometer system is used background can be as low as 25-50 counts per minute.

Higher Sensitivity. The larger volume of the 3" crystals significantly increases their efficiency. Efficiency for I-131 gammas (.364 Mev) is approximately 68% as contrasted with an I-131 efficiency of 50% or less with conventional 1⅞" diameter well crystals. Efficiency is even more marked when high energy gamma emitters, such as cobalt-60 (1.33 and 1.17 Mev), are counted. A conventional 1⅞" crystal has an efficiency of about 38% for Co-60 gammas, while the 3" crystal offers an efficiency of more than 60%.

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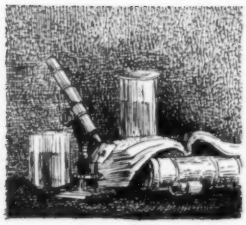
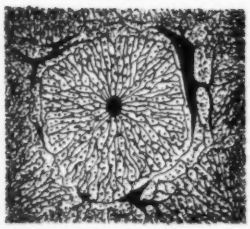
IT HAPPENED THIS MONTH...

a glance at yesterday in relation to today



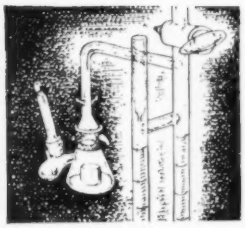
IN MARCH—(1884)—*Science* reviews a Brazilian pamphlet on the genesis of beriberi. Dr. J. B. DeLacerda cultured blood of beriberi patients in meat solution and obtained a microphyte similar to the "bacillus of carbuncle". Animals infected with this organism succumb to paralytic disease in 5 to 20 days. The microphyte was then recovered from the muscles and spinal medulla. "From these facts the author draws the logical conclusion, that beriberi is a parasitic disease, and that the parasites attack particularly the blood, muscles, and medulla."[1]

With the help of an increased fund of knowledge, we now know that beriberi results from deficiency of cocarboxylase (thiamine pyrophosphate), a derivative of vitamin B_1. Schwarz BioResearch is a leading supplier of cocarboxylase, as well as other important metabolic cofactors such as ATP, ADP, adenylic acid, flavin adenine dinucleotide, DPN, guanosine diphosphate and guanosine triphosphate. Many of these have been labeled with C^{14}.



IN MARCH—(1921)—Frederick Gowland Hopkins reports the discovery of a new sulfhydryl compound in yeast, liver, and muscle cells.[2] This compound appears to contain a dipeptide of glutamic acid and cystein. Although present in low concentration, it accounts for almost all the non-protein organically bound sulfur in the cell. The cystein moiety is found to change readily from the sulfhydryl to the disulfide state under the influence of factors shown to be present in the tissues.

In the intervening 40 years, we have learned that the compound actually contained a third amino acid, glycine, and was the tripeptide we now call glutathione. *If you are interested in carrying still further an investigation of the chemical or biological properties of glutathione, Schwarz BioResearch can supply you with both the oxidized and reduced forms—plain or labeled with S^{35} or C^{14}. We can also provide you with the common sulfur amino acids and many of their derivatives. Write for our latest catalog and price list.*



IN MARCH—(1949)—Otto Meyerhof publishes another of his interesting papers in the field of carbohydrate metabolism. Previous studies had shown that large amounts of phosphorylated hexoses are required to maintain steady continuous glycolysis in homogenates or centrifuged extracts of malignant tumors. In the absence of such phosphate donors, continuous glucose utilization can occur only if the activity of ATPase (in terms of phosphate turnover) is twice that of hexokinase. Meyerhof and Wilson[3] show that enzymatic balance may be obtained by adding yeast hexokinase or inhibiting the activity of the tumor ATPase.

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1. (Review): The bacillus of beriberi. Science *3*:331 (March 14) 1884. 2. Hopkins, F. G.: On an autoxidisable constituent of the cell. Biochem J. *15*:286 (1921). 3. Meyerhof, O. and Wilson, J. R.: Studies on the enzymatic system of tumor glycolysis. I. Glycolysis of free sugar in homogenates and extracts of transplanted rat sarcoma. Arch. Biochem. *21*:1 (March) 1949.

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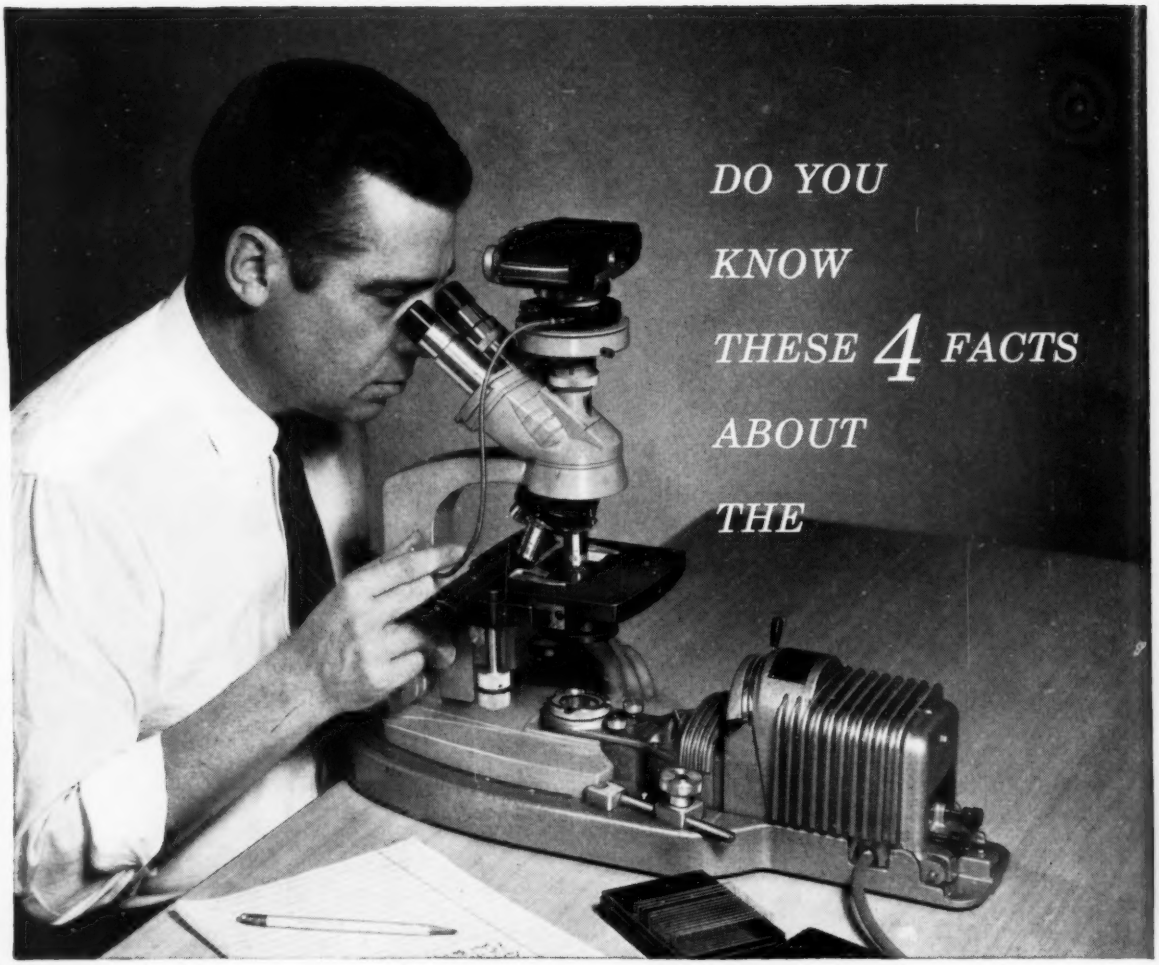

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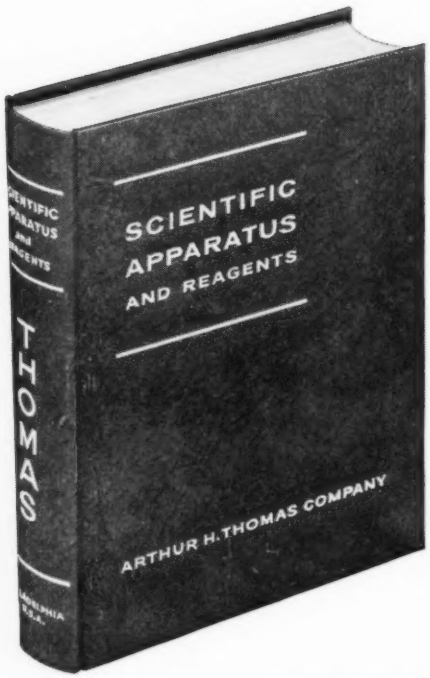

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The Journal of Laboratory and Clinical Medicine

VOL. 57 MARCH, 1961 NO. 3

CLINICAL AND EXPERIMENTAL

EFFECT OF DIETARY CHOLESTEROL UPON SERUM LIPIDS IN MAN

WILLIAM E. CONNOR, M.D., ROBERT E. HODGES, M.D., AND
ROBERTA E. BLEILER, PH.D.
IOWA CITY, IOWA

It is a common assumption that dietary cholesterol has little influence upon human serum cholesterol levels; yet cholesterol intake has a great effect in animals. This problem was studied again in six men who consumed alternatively diets high in egg yolk (providing 1,650 to 4,800 mg. of cholesterol) and cholesterol-free diets. The iodine numbers and amounts of saturated, monoethenoid, and polyethenoid fatty acids in the high cholesterol and cholesterol-free diets were similar, so that effects from differences in dietary fat were minimized.

The mean serum cholesterol in three subjects increased 57 mg. per cent when cholesterol was added to the cholesterol-free diet. It declined 102 mg. per cent in the three other subjects during the period of the cholesterol-free diet. The serum phospholipid changed with the serum cholesterol. An increase in the polyethenoid fatty acid content of the diet with a concomitant decrease in the saturated fatty acids failed to lower the serum cholesterol in subjects simultaneously given the high cholesterol diet. Neither dietary protein within a range of 20 to 143 Gm. per day nor the nitrogen balance affected the serum lipid levels.

CHOLESTEROL consumed in the human diet is usually considered to have little influence upon the serum cholesterol level.[2] This belief has led logically to the conclusion that dietary cholesterol does not contribute to the pathogenesis of atherosclerosis in man and need not be considered in its therapy.[3]

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In experimental animals the opposite holds; for here cholesterol *is* almost always an essential component of diets designed to cause hypercholesteremia and atherosclerosis.[4] Recently the human disease has been mimicked closely in the monkey by prolonged feeding of a high cholesterol diet with 22 per cent butter fat which produced extensive coronary atherosclerosis and myocardial infarction.[5] This apparent difference in the response of man and other animals to dietary cholesterol has led us to study the problem anew.

Previous work concerned with the effect of dietary cholesterol upon the serum cholesterol has taken two forms: crystalline cholesterol has been fed directly or a natural food that contains a high concentration of cholesterol such as egg yolk has been given. The ingestion of even large amounts of crystalline cholesterol has usually failed to induce significant increases in the serum cholesterol.[2] On the other hand, the feeding of cholesterol in the form of egg yolk has led to increased serum values. However, many of the egg yolk studies are difficult to interpret in the light of the present-day knowledge that the amount and kind of fat in the diet have a profound influence upon the serum cholesterol level.[6] For example, in 1933 Okey and Stewart[7] obtained a rise in the mean serum cholesterol level of young women when egg yolks were added to a low cholesterol, low fat diet. Both the fat and cholesterol content of this diet were increased. Steiner and Domanski[8] gave ten patients 2.4 Gm. of cholesterol in the form of egg yolk daily for 6 to 10 weeks and obtained an average increase of 102 mg. per 100 ml. in the serum cholesterol concentration. Again, the addition of egg yolk increased the fat content of the diet by 67 Gm. per day. In a later study, Messinger and associates[9] found that a diet containing 3.75 Gm. of cholesterol per day from egg yolk resulted in an increase in the serum cholesterol from 27 to 61 mg. per 100 ml., whereas crystalline cholesterol, 30 Gm. per day, had little effect. However, the fat contents of the control and experimental diets were not balanced. Bronte-Stewart and co-workers[10] obtained a pronounced rise in the serum cholesterol of 60 mg. per 100 ml. when 10 eggs replaced the hydrogenated groundnut fat (iodine value of 55) of a cholesterol-free diet. The data of this experiment might be interpreted to indicate an effect from dietary cholesterol since the fatty acids of eggs are more unsaturated than those of hydrogenated groundnut oil and would have otherwise caused a fall in serum cholesterol.

Other approaches to the relationship of dietary cholesterol and the serum cholesterol have involved the removal of cholesterol from the diet. The rice and fruit diet invariably lowered the serum cholesterol[2] but this diet is low in fat and protein as well as in cholesterol. The shift from the usual American diet to a "strict" vegetarian diet completely eliminates dietary cholesterol. Such a diet[11, 12] and vegetarianism in a population group[13] clearly led to lower serum cholesterol levels. Since the dietary fat is concomitantly either reduced in amount or increased in its unsaturation as animal fats are eliminated, the vegetarian diet might have effects from both its altered fat and its cholesterol content.

In the study to be described we wished to investigate the effects of *feeding* or *not feeding* cholesterol as found in a natural and commonly eaten food, i.e.,

egg yolk. We have given special concern to balancing the calories, protein, and fat of the different experimental diets. Furthermore, an attempt has been made to equalize the iodine numbers and fatty acid compositions of the high cholesterol and cholesterol-free diets, so that effects from dietary fat changes which might alter the serum cholesterol could be largely excluded in this study.

TABLE I. PLAN OF STUDY

SUBJECTS	PERIOD AND DIET			
	I (35 DAYS)	II (75 DAYS)	III (23 DAYS)	IV (17 DAYS)
Group A (1, 2, 3)	General	High cholesterol	Cholesterol-free	General
Group B (4, 5, 6)	General	Cholesterol-free	High cholesterol	General

METHODS

Subjects.—Six healthy men were selected from a group of volunteering prisoners and housed on a metabolism ward for a 150 day period. Their ages ranged from 40 to 45 years. Four subjects were well nourished and two subjects (Subjects 1 and 4) were overweight, about 20 and 35 pounds, respectively. Subject 1 had xanthelasma, 4 mm. in diameter, on the lower lids. The medical histories and physical examinations of all subjects were otherwise normal. The urine, blood hemoglobin, white blood cell counts, and chest x-rays were normal. All subjects remained in good health for the duration of the study.

Dietary Plan.—The subjects were divided into two groups of three men each, Groups A and B. Each group of men received a different diet for four distinct dietary periods (Table I). For Period I all subjects received a general diet, the composition of which is shown in Table II. The 35 days of Period I were utilized to acquaint the men with their surroundings, to allow adjustment of body economy to a new diet (a change from the previous prison fare), and to accustom them to venipunctures and collections of stool and urine for balance studies.

The diet of Period II was a formula diet drunk from a cup. It was complete in all known nutrients (Table III). The formulas for the three subjects in Group A contained egg yolk; the three Group B subjects received no egg yolk. The protein content was adjusted by the use of skim milk with or without egg yolk so that paired subjects, one from each group, received the following: 20 Gm. of protein per day (Subjects 1 and 4), 1 Gm. of protein per kilogram of body weight (Subjects 2 and 5), and 2 Gm. of protein per kilogram of body weight (Subjects 3 and 6). The sources of fat were egg yolk and peanut oil for Group A but only peanut oil for Group B. The amount of fat, the iodine number, and the fatty acid composition of the diet given each subject are shown in Tables II and IV. Thus the three subjects in Group A had a "high cholesterol" diet containing 1,650, 1,900, and 4,800 mg. of cholesterol in the form of egg yolks (119, 136, and 340 Gm., respectively). Subjects in Group B were given a "cholesterol-free" diet. Carbohydrate was supplied by skim-milk solids plus lactose, Dextri-Maltose No. 1, dextrose, and cornstarch to make up the requisite calories for each subject. Supplemental vitamins and minerals were added to the formula so that the recommended daily allowances of the National Research Council were met or exceeded and so that each subject received comparable amounts of these substances. Cystine was added to maintain a tryptophane to cystine and methionine ratio of about 1:4. Subjects 1 and 6 developed a mild diarrhea which did not influence their nitrogen balances, and Subjects 3 and 4 became constipated.

In Period III Group A was changed from the high cholesterol diet to a cholesterol-free diet and Group B from the cholesterol-free diet to a diet containing 2,400 mg. cholesterol per day, supplied by egg yolks. The diet of this period consisted of a variety of natural solid foods. All subjects ate a diet high in protein (114 to 139 Gm. per day) during this period.

The fat consumed by both groups was equivalent in amount and similar in iodine number and fatty acid composition (Tables II and IV). It was derived almost entirely from

TABLE II. DIET, BODY WEIGHT, AND NITROGEN BALANCE FOR EACH SUBJECT BY PERIODS

SUBJECT	DIETARY PERIODS	DIET						INITIAL WEIGHT (LB.)	WEIGHT CHANGE (LB.)	NITROGEN BALANCE (GM./DAY)
		CALORIES	PROTEIN (GM.)	FAT (GM.)	FAT: % OF TOTAL CALORIES	IODINE NO.	CHOLESTEROL* (MG.)			
Group A.—										
1	I General	2,945	126	125	38	66	950	185	0	+0.48
	II High cholesterol	2,908	20	132	41	88-94	1,650	185	−15	−0.37
	III Cholesterol-free	2,884	115	132	41	89-92	0	170	+4	+0.61
	IV General	3,603	131	179	45	56	1,400	174	+8	
2	I General	2,519	120	107	38	69	900	127	+1	+0.41
	II High cholesterol	2,005	58	90	40	86-93	1,900	128	−12	+0.22
	III Cholesterol-free	2,470	114	114	42	86-89	0	116	−2	+0.17
	IV General	3,485	133	173	45	57	1,100	114	+7	
3	I General	2,519	120	107	38	69	900	155	−1	+0.43
	II High cholesterol	2,492	143	111	40	80-90	4,800	154	−3	+0.40
	III Cholesterol-free	2,470	115	114	41	87-90	0	151	−4	−0.05
	IV General	3,363	126	163	44	55	1,100	147	+5	
Group B.—										
4	I General	2,439	120	107	40	69	900	219	+1	+0.41
	II Cholesterol-free	2,480	20	111	40	92-96	0	220	−17	−0.32
	III High cholesterol	2,452	118	116	43	82-89	2,400	203	+4	+0.28
	IV General	3,375	124	163	44	52	1,100	207	+5	
5	I General	2,945	126	125	38	66	950	173	0	+0.38
	II Cholesterol-free	2,935	72	132	40	92-96	0	173	0	−0.04
	III High cholesterol	2,943	120	139	42	84-89	2,400	173	+1	−0.06
	IV General	3,524	126	174	44	55	1,200	174	+5	
6	I General	2,659	120	107	36	66	900	154	−2	+0.69
	II Cholesterol-free	2,689	143	120	40	92-96	0	152	−2	+0.23
	III High cholesterol	2,676	119	124	42	85-90	2,400	151	+2	+0.06
	IV General	2,970	104	140	42	57	1,000	153	+2	

*Computed on the basis of values given by Hardinge and Crooks.[18]

peanut oil, peanuts, peanut butter, or egg yolk. Only Group B subjects received egg yolk as a part of the fat intake. The duration of Period III was 23 days.

The study was concluded with a general diet during which each subject was allowed a free choice of menu (Period IV). The food intake was recorded.

Laboratory Studies.—Blood was drawn twice weekly from each subject after a 14 hour fast. The total cholesterol,[14] phospholipid,[15] and triglyceride[16] were determined in duplicate on each serum specimen during all periods. Nitrogen was determined on all food intake and on all stool and urine specimens during Periods I, II, and III, and the nitrogen balance was calculated for each subject. A modification of the Scales and Harrison method was used for this determination.[17]

TABLE III. FORMULAS FOR PERIOD II. AMOUNTS OF NUTRIENTS IN GRAMS

SOURCE	SUBJECTS					
	1	2	3	4	5	6
Protein.—						
Dried skim milk solids		34	55	19	70	140
Egg yolk	19	22	85			
Cystine	0.3	1.2	3.0	0.4	1.5	3.0
Fat.—						
Dried skim milk solids		1	2	0.5	2	4
Egg yolk	38	43	109			
Peanut oil	94	44		110	130	116

The intake of lactose was 200 Gm. per man per day. Dextrose, Dextri-maltose No. 1, and cornstarch were added to each formula to meet calorie needs.

Vitamin A was 10,000 to 11,000 U.S.P. units and vitamin D, 500 U.S.P. units. Other vitamins, in the form of a vitamin B complex liquid and ascorbic acid, were added to meet recommended dietary allowances of the Food and Nutrition Board of the National Research Council.

The mineral content of each formula was similar: sodium, 2.75 Gm.; potassium, 5.7 Gm.; calcium, 5.0 Gm.; phosphorus, 4.31 Gm.; iron, 26 mg.; magnesium, 500 mg.; iodine, 0.1 mg.

TABLE IV. THE FATTY ACID COMPOSITION OF THE DIETS FOR EACH SUBJECT* (BY PER CENT)

SUBJECT	PERIOD II			PERIOD III		
	SATURATED	MONOETHENOID	POLYETHENOID	SATURATED	MONOETHENOID	POLYETHENOID
Group A.—						
1	24	50	26	21	53	25
2	26	47	27	22	53	25
3	32	40	27	21	53	26
Group B.—						
4	20	54	26	26	47	27
5	20	54	26	26	48	26
6	20	54	26	26	47	26

*The computations were made on the basis of the fatty acid compositions given by Hardinge and Crooks.[18] The fats consumed during Period II were egg yolk and peanut oil. The fats for Period III were egg yolk, peanut oil, and peanut butter (partially hydrogenated).

RESULTS

Fig. 1 illustrates the individual serum cholesterol values for each subject during the four dietary periods of the study. During the first general diet period, Subject 1 was hypercholesteremic with a mean of 280 mg. per 100 ml. The five other subjects had mean values ranging from 200 to 248 mg. per 100 ml. During Period II, 3 weeks after the shift to the high cholesterol diet for Group A and to the cholesterol-free diet for Group B, a distinct separation of

TABLE V. MEAN SERUM LIPID VALUES* FOR DIFFERENT DIETARY PERIODS (MG. PER 100 ML.)

SUBJECT	DIETARY PERIODS	CHOLESTEROL				PHOSPHOLIPID				TRIGLYCERIDE			
		INITIAL	FINAL	RANGE	CHANGE	INITIAL	FINAL	RANGE	CHANGE	INITIAL	FINAL	RANGE	CHANGE
Group A.—													
1	I General		280	271-290			222	203-244			201	179-218	
	II High cholesterol†	280	316	303-325	36	222	247	228-265	25	201	296	270-370	95
	III Cholesterol-free	316	222	218-225	−94	247	213	212-213	−34	296	165	158-171	−131
	IV General	222	279	270-287	55	213	226	221-230	13	165	179	174-183	14
2	I General		248	233-258			217	200-230			82	65-93	
	II High cholesterol	248	273	250-295	25	217	233	213-250	16	82	98	87-110	16
	III Cholesterol-free	273	158	155-161	−115	233	168	162-174	−65	98	63	61-64	−35
	IV General	158	235	229-240	77	168	217	209-225	67	63	78	77-78	15
3	I General		223	208-238			182	171-190			81	73-85	
	II High cholesterol‡	223	253	246-270	30	182	204	181-228	22	81	75	62-89	−4
	III Cholesterol-free	253	155	151-159	−98	204	157	156-157	−47	75	58	53-63	−17
	IV General	155	222	218-226	67	157	192	190-194	35	58	76	75-76	18
Group B.—													
4	I General		239	225-263			216	206-225			161	137-202	
	II Cholesterol-free†	239	178	161-190	−61	216	166	163-169	−50	161	138	116-151	−23
	III High cholesterol	178	258	251-264	80	166	224	223-224	58	138	139	134-144	1
	IV General	258	221	220-221	−37	224	213	206-219	−11	139	117	108-125	−22
5	I General		200	180-219			183	162-191			171	148-183	
	II Cholesterol-free	200	156	128-168	−44	183	159	145-169	−24	171	147	108-210	−24
	III High cholesterol	156	171	169-173	15	159	170	166-173	11	147	176	157-182	29
	IV General	171	192	187-197	21	170	204	198-209	34	176	145	127-162	−31
6	I General		219	211-227			206	194-219			71	63-78	
	II Cholesterol-free‡	219	135	120-150	−84	206	166	138-163	−40	71	50	42-56	−21
	III High cholesterol	135	211	198-223	76	166	213	206-220	47	50	67	66-68	17
	IV General	211	193	187-199	−18	213	192	190-194	−21	67	74	73-74	7

*Mean values were computed on the basis of results obtained over the last 14 days of Periods I and II and the last 7 days of Period III and IV.

†Subjects 1 and 4 received only 20 Gm. of protein per day.

‡Subjects 3 and 6 received a high protein diet (2 Gm. per kilogram of body weight per day).

the serum cholesterol levels of the two groups was evident. These changes were consistent over the entire 75 days of Period II. The three subjects of Group A had mean increases of serum cholesterol ranging from 25 to 36 mg. per 100 ml. whereas the Group B subjects on the cholesterol-free diet had decrements of from 44 to 84 mg. per 100 ml. (Table V and Fig. 1). The serum phospholipid rose 16 to 25 mg. in Group A and fell 24 to 50 mg. per 100 ml. in Group. B. All subjects in Group B had a decrease of the serum triglyceride, but only Subjects 1 and 2 of Group A had increased triglyceride. Subject 3 had no change.

When the diets were interchanged for each group in Period III the serum lipids shifted correspondingly (Table V and Fig. 2). All subjects of Group A changed from the high cholesterol to the cholesterol-free diet had pronounced decrements in the serum cholesterol values ranging from 94 to 115 mg. per 100 ml. The phospholipid fell 34 to 65 mg. per 100 ml. and the triglyceride declined 17 to 131 mg. per 100 ml. The subjects of Group B, previously receiving the cholesterol-free diet, had increases in the serum lipids while they were on the high cholesterol diet. The serum cholesterol rose from 15 to 80 mg. per 100 ml. and the serum phospholipid increased from 11 to 58 mg. per 100 ml. The serum triglyceride values increased in Subjects 5 and 6 but did not change in Subject 4.

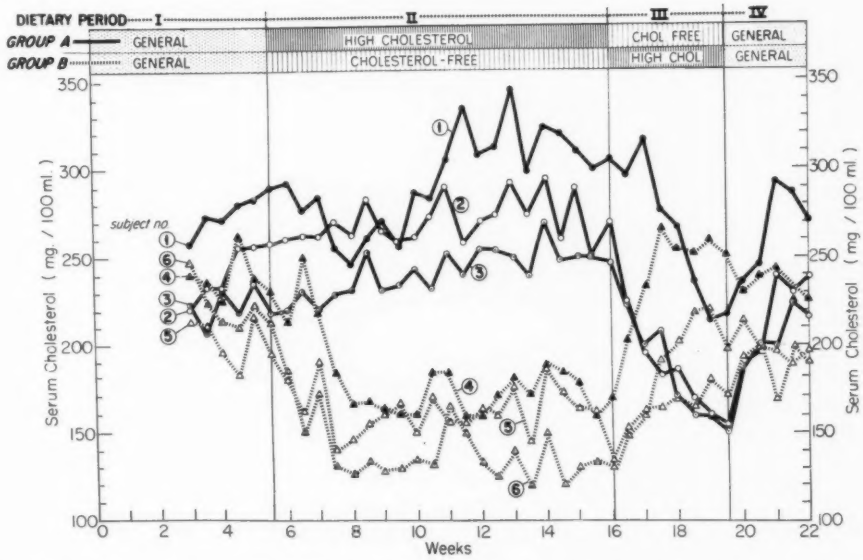

Fig. 1.—The serum cholesterol values for each of the six subjects during all four dietary periods.

The ad libitum general diet of Period IV caused the serum lipids of Group A subjects to increase greatly so that all values became similar to those obtained during the general diet of Period I. The serum cholesterol rose 55 to 77 mg. per 100 ml., the serum phospholipid increased 13 to 67 mg., and the serum triglyceride only 14 to 18 mg. Group B subjects had variable shifts in changing from the high cholesterol diet to the ad libitum general diet. Subjects 4 and 6

had declines in the serum cholesterol and phospholipid levels, whereas the values of Subject 5 continued to climb. Triglyceride changes were inconsistent.

Subjects 1 and 2 of Group A lost 15 and 12 pounds of weight, respectively, during the 75 days of Period II when they received the high cholesterol formula diet (Table II). Despite this weight loss their serum cholesterol levels increased 36 and 25 mg. per 100 ml. (Table V). Their serum phospholipid and triglyceride levels also rose in this period. Subject 4 of Group B lost 17 pounds while receiving the cholesterol-free diet (Period II). His serum cholesterol level fell 61 mg. per 100 ml.; the other lipids declined similarly. Two of these subjects had difficulty at times in consuming all of the formula; the other (Subject 2) received insufficient calories for his needs. Because some subjects of both groups lost weight, it seemed that weight reduction in this study did not seem to be a factor in producing lowering of the serum cholesterol.

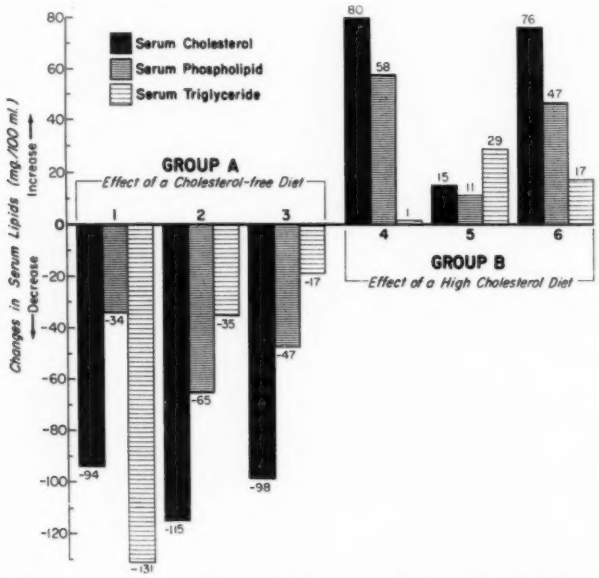

Fig. 2.—The changes in serum lipids which occurred in each subject when the diets were altered from Period II to Period III. For Group A the change was from a high cholesterol diet to the cholesterol-free diet. For Group B the change was from a cholesterol-free diet to the high cholesterol diet.

Effect of Different Levels of Protein Intake and Nitrogen Balance.—Two subjects received only 20 Gm. of protein per day for the 75 days of Period II. Their average daily negative nitrogen balances were 0.37 Gm. for Subject 1 and 0.32 Gm. for Subject 4 (Table II). Subject 1, who was given the high cholesterol diet, had distinct elevations of the serum cholesterol, phospholipid, and triglyceride during this period of negative nitrogen balance. Subject 4, who received the cholesterol-free diet, had the same pronounced fall in the serum lipids as did the other subjects in Group B who had adequate protein intake and were in positive nitrogen balance. The administration of a high protein diet, 2 Gm. per kilogram of body weight, to Subjects 3 and 6 led to positive nitrogen balances but did not affect the serum lipids. The serum cholesterol of

Subject 3 increased and the serum cholesterol of Subject 6 declined greatly. Thus the serum lipid changes for the subjects in this study did not correlate with the quantity of dietary protein ingested over the range from 20 to 143 Gm. per day.

DISCUSSION

This study shows that dietary cholesterol given as egg yolk produced a pronounced elevation of serum cholesterol and phospholipid values. This effect has been demonstrated by the use of different experimental diets containing fats equivalent in amount and similar in iodine number and fatty acid composition but vastly different in cholesterol content. Essentially we have compared egg yolk lipids versus peanut lipids with diets otherwise similar in the quantity of fat and in calories, carbohydrate, and protein. The differential effect of egg lipids versus peanut lipids upon serum cholesterol levels might result from an "elevating factor" in egg yolk or a "depressant factor" in peanut lipids. Besides the presence or absence of cholesterol, other constituents of these two dietary substances might conceivably have an influence upon serum lipid levels. These include triglyceride, phospholipid, and sitosterol.

The fatty acid compositions of egg yolk and peanut oil are similar in that both contain long chain fatty acids. Typical analyses report that egg yolk contains somewhat more saturated fatty acids and less oleic acid than peanut oil, and a similar amount of polyethenoid fatty acids.[18] Deuel[19] gives the iodine number of egg yolk lipids as between 80 and 90. The iodine number of peanut oil varies from 92 to 96.[20]

It is doubtful that these small differences in fatty acid content could explain the pronounced effects upon serum cholesterol levels found in this study. All but one of the high cholesterol diets employed in our study during Periods II and III contained both peanut oil and egg yolk. Thus the fatty acid composition of the high cholesterol and cholesterol-free diets were similar (Table IV). Subject 3, who received only egg yolk lipid during Period II, had serum cholesterol changes similar to those of the other subjects consuming both egg yolk and peanut oil lipids. The feeding of fats having iodine values differing only slightly (a difference of from 10 to 20) has led to similar serum cholesterol responses.[6, 21] Even when egg yolks containing more unsaturated fatty acids (iodine number of 100) were fed to humans, their usual hypercholesterolemic effect was not altered.[22]

From the serum cholesterol changes which occurred in the subjects of Group A as their diet was changed from the general to the high cholesterol diet (Period I to Period II), it is clear that dietary cholesterol may counteract an expected hypocholesterolemic action of dietary fats. In this dietary shift the iodine number of the diet was increased from 66 through 69 to 80 through 94. If only the changes in dietary fat composition were being considered, it might be expected that the serum cholesterol of these subjects would decline somewhat, since the unsaturation of the diet was being increased. Instead they developed increased serum cholesterol concentrations. Only Group B subjects, receiving the cholesterol-free diet during Period II, had greatly lowered serum cholesterol values.

These decrements of 44 to 84 mg. per 100 ml. were greater than might be predicted from the change in dietary fat composition.[6, 21] Thus, for both groups in the dietary changes from Period I to Period II, the alterations in serum cholesterol reflected the presence or absence of dietary cholesterol as well as the change in fat composition.

In addition to cholesterol and fatty acids, phosphatide is another constituent of egg yolk which might affect the serum cholesterol level. Egg yolk is rich in phosphatide, 8 to 14 per cent by weight, of which the choline containing lecithin is in greatest concentration.[23] By contrast, whole peanuts contain only 2.2 per cent phosphatide, and peanut oil has no phosphatide. Since the intestinal tract appears to absorb phosphatides as the constituent fatty acids of a given phosphatide,[24] one effect of the ingestion of egg yolk phosphatide might be from its content of fatty acids. These resemble the fatty acids of egg yolk triglyceride except for somewhat greater unsaturation. With regard to the choline constituent there are many reports that choline has failed to influence human serum cholesterol values.[25]

It is unlikely that the sitosterol content of peanut oil would contribute to the hypocholesterolemia caused by the change from the high cholesterol diet to the cholesterol-free diet. Peanut oil contains plant sterols in a concentration of 0.25 Gm. per 100 ml.[20] While ingesting peanut oil none of our subjects received more than 0.32 Gm. of plant sterols per day. Many of the subjects eating the high cholesterol diet also received considerable peanut oil and, hence, sitosterols. For example, Subject I in Period II on the high cholesterol diet consumed 0.23 Gm. of sitosterols as compared with 0.27 Gm. eaten by Subjects 4, 5, and 6 on the cholesterol-free diet. Similarly, in Period III, all subjects on the high cholesterol diet also received sitosterols. The total daily amounts consumed by all subjects were small compared with the reported 10 Gm. of sitosterol per day which must be taken to affect the serum cholesterol concentrations.[26]

The dietary cholesterol consumed during the two crucial experimental periods (II and III) of this study ranged from virtually no cholesterol to intakes of 1,650 to 4,800 mg. for subjects receiving the high cholesterol diet. The amounts of cholesterol in these high cholesterol diets provided a greater intake than the 400 to 1,200 mg. range which many human diets contain. No differences in serum lipid responses were noted for the varied cholesterol intakes of subjects receiving a high cholesterol diet. It seems likely that the gastrointestinal tract has a limited ability to absorb dietary cholesterol, all cholesterol above a certain percentage of the intake being passed in the feces.[27] Possibly at higher levels of intake the percentage absorbed is less. Only further investigation will determine the critical level of cholesterol intake below which the high cholesterol diet does not have a hypercholesteremic effect. Our studies suggest that this critical level is certainly below 1,650 mg. per day. The epidemiologic evidence indicates that it may be lower than the range of 220 to 400 mg. per day,[2] perhaps varying from one individual to another.

Most investigators have found that the feeding of crystalline cholesterol to humans has not produced significant increases in the serum cholesterol

levels.[2, 6, 8-10, 22] An exception is the recent work of Beveridge and co-workers,[28] who fed volunteers formulas containing various distilled butter fat fractions over a short period of time. The serum cholesterol rose when crystalline cholesterol was added to a butter fat fraction low in cholesterol. Cook[27] has found that crystalline cholesterol dissolved in a vegetable oil is poorly absorbed from the gastrointestinal tract. On the other hand, the absorption of egg yolk cholesterol is four times more complete. Perhaps the physical state of cholesterol in egg yolk as lipoprotein complexes accounts for its greater intestinal absorption.

Dietary Protein and Serum Cholesterol.—This study did not show a relationship between the amount of dietary protein in the range of 20 to 143 Gm. per day and the serum cholesterol response. The nitrogen balance also did not seem to affect the serum cholesterol level. Olson and associates[29] had obtained a lowering in serum cholesterol with a low protein diet deficient in amino acids and choline. The low protein, egg yolk diet of our Subject 1 was adequate in amino acids and high in choline. A similar lack of effect from the ingestion of a very high protein diet had also been found by Keys and Anderson.[30]

SUMMARY

1. Six subjects consumed alternately diets high in cholesterol from egg yolk and cholesterol-free diets in a study of 150 days' duration. The serum cholesterol and phospholipid levels rose when subjects consumed the high cholesterol diet and declined markedly when the cholesterol-free diet was eaten. The serum triglyceride levels less consistently tended to rise and fall with the presence or absence of cholesterol.

2. The iodine numbers and the amounts of saturated, monoethenoid, and polyethenoid fatty acids in the high cholesterol and cholesterol-free diets were similar. Thus effects from differences in dietary fat composition were minimized in this study. Likewise, differences in phosphatide and sitosterol content did not appear to affect the results.

3. An increase in the polyethenoid fatty acid content of the diet with a concomitant decrease in the saturated fatty acids failed to lower the serum cholesterol in subjects simultaneously given a high cholesterol diet.

4. Levels of dietary protein from 20 Gm. to 143 Gm. per day did not influence the serum lipid levels. The serum lipids were not altered by either a positive or negative nitrogen balance.

REFERENCES

1. Connor, W. E., and Hodges, R. E.: Effect of Dietary Cholesterol Upon Serum Lipids in Man, J. Lab. & Clin. Med. **54**: 800, 1959. (Abst.)
2. Keys, A., Anderson, J. T., Mickelsen, O., Adelson, S. F., and Fidanza, F.: Diet and Serum Cholesterol in Man: Lack of Effect of Dietary Cholesterol, J. Nutrition **59**: 39, 1956.
3. Keys, A., and Anderson, J. T.: The Relationship of the Diet to the Development of Atherosclerosis in Man, *in* Symposium on Atherosclerosis, Monograph Publication 338, Washington, D. C., 1954, National Research Council, p. 182.
4. Katz, L. N., and Stamler, J.: Experimental Atherosclerosis, Springfield, Ill., 1953, Charles C Thomas, Publisher.
5. Taylor, C. B., Cox, G. E., Counts, M., and Yogi, N.: Fatal Myocardial Infarction in Rhesus Monkeys With Diet-Induced Hypercholesterolemia, Circulation **20**: 975, 1959.
6. Ahrens, E. H., Jr., Hirsch, J., Insull, W., Tsaltas, T. T., Blomstrand, R., and Peterson, M. L.: The Influence of Dietary Fats on Serum-lipids Levels in Man, Lancet **1**: 943, 1957.

7. Okey, R., and Stewart, D.: Diet and Blood Cholesterol in Normal Women, J. Biol. Chem. **99**: 717, 1933.
8. Steiner, A., and Domanski, B.: Dietary Hypercholesteremia, Am. J. M. Sc. **201**: 820, 1941.
9. Messinger, W. J., Porosowska, Y., and Steele, J. M.: Effect of Feeding Egg Yolk and Cholesterol on Serum Cholesterol Levels, Arch. Int. Med. **86**: 189, 1950.
10. Bronte-Stewart, B., Antonis, A., Eales, L., and Brock, J. F.: Effects of Feeding Different Fats on Serum-Cholesterol Level, Lancet **1**: 521, 1956.
11. Ahrens, E. H., Jr., Hirsch, J., Insull, W., Jr., Tsaltas, T. T., and Blomstrand, R.: Dietary Control of Serum Lipids in Relation to Atherosclerosis, J. A. M. A. **164**: 1905, 1957.
12. Groen, J., Tjiong, B. K., Kamminga, C. E., and Willebrands, A. F.: The Influence of Nutrition, Individuality and Some Other Factors, Including Various Forms of Stress, on the Serum Cholesterol, an Experiment of Nine Months Duration, Voeding **13**: 556, 1952.
13. Hardinge, M. G., and Stare, F. J.: Nutritional Studies of Vegetarians. I and II, Am. J. Clin. Nutrition **2**: 73, 1954.
14. Zak, B.: Simple Rapid Microtechnic for Serum Total Cholesterol, Am. J. Clin. Path. **27**: 583, 1957.
15. Dryer, R. L., Tammes, A. R., and Routh, J. I.: The Determination of Phosphorus and Phosphatase With N-Phenyl-p-phenylene Diamine, J. Biol. Chem. **225**: 177, 1957.
16. Van Handel, E., and Zilversmit, D. B.: Micromethod for the Direct Determination of Serum Triglycerides, J. Lab. & Clin. Med. **50**: 152, 1957.
17. Scales, F. M., and Harrison, A. P.: Boric Acid Modification of the Kjeldahl Method for Crop and Soil Analysis, J. Indust. Eng. Chem. **12**: 350, 1920.
18. Hardinge, M. G., and Crooks, H.: Fatty Acid Composition of Food Fats, J. Am. Dietet. A. **34**: 1065, 1958.
19. Deuel, H. J., Jr.: The Lipids: Their Chemistry and Biochemistry, vol. 2, Biochemistry, New York, 1955, Interscience Publishers, Inc., p. 811.
20. Hoffpauir, C. L.: Peanut Composition. Relation to Processing and Utilization, Agricultural & Food Chem. **10**: 668, 1953.
21. Keys, A., Anderson, J. T., and Grande, F.: Prediction of Serum-Cholesterol Responses of Man to Changes in Fats in the Diet, Lancet **2**: 959, 1957.
22. Gordon, H., Wilkens, J., and Brock, J. F.: Serum Cholesterol Levels After Consuming Eggs With Increased Content of Unsaturated Lipids, Lancet **2**: 244, 1958.
23. Wittcoff, H.: The Phosphatides, New York, 1951, Reinhold Publishing Corporation.
24. Blomstrand, R., Thorn, N. A., and Ahrens, E. H., Jr.: The Absorption of Fats Studied in a Patient With Chyluria, Am. J. Med. **24**: 958, 1958.
25. Kritchevsky, D.: Cholesterol, New York, 1958, John Wiley & Sons, Inc., p. 207.
26. Joyner, C. R., Jr., and Kuo, P. T.: Effect of Sitosterol Administration Upon Serum Cholesterol Level and Lipoprotein Pattern, Am. J. M. Sc. **230**: 636, 1955.
27. Cook, R. P., Edwards, D. C., and Riddell, C.: Cholesterol Metabolism; Cholesterol Absorption and Excretion in Man, Biochem. J. **62**: 225, 1956.
28. Beveridge, J. M. R., Connell, W. F., Haust, H. L., and Mayer, G. A.: Dietary Cholesterol and Plasma Cholesterol Levels in Man, Canad. J. Biochem. Physiol. **37**: 575, 1959.
29. Olson, R. E., Vester, J. W., Gursey, D., Davis, N., and Longman, D.: The Effect of Low-Protein Diets Upon the Serum Cholesterol in Man, Am. J. Clin. Nutrition **6**: 310, 1958.
30. Keys, A., and Anderson, J. T.: Dietary Protein and the Serum Cholesterol in Man, Am. J. Clin. Nutrition **5**: 29, 1957.

ERYTHROCYTE GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY RELATED TO THYROID ACTIVITY

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Erythrocyte glucose-6-phosphate dehydrogenase (G-6-P. dehydrogenase) has been investigated with respect to thyroid activity. Our normal value of G-6-P. dehydrogenase is 137 ± 18.9 enzyme units per 100 ml. of red blood cells. Eleven of twelve patients with thyrotoxicosis had elevated enzyme levels averaging 232 ± 39 enzyme units per 100 ml. of red blood cells. This difference is highly significant ($p < 0.001$). None of the patients were anemic and their reticulocyte counts were normal. Two patients with high enzyme levels had normal plasma iron turnovers, suggesting that increased erythropoietic activity was not responsible for the increase of G-6-P. dehydrogenase. Administration of thyroxine and tri-iodothyronine to a normal person was associated with a rapid increase in G-6-P. dehydrogenase levels. In vitro incubation of red blood cells and tri-iodothyronine showed no augmentation of enzyme activity. Four patients with hypothyroidism had normal enzyme levels. A direct relationship between increased thyroid activity and red blood cells G-6-P. dehydrogenase is demonstrated in vivo. However, the mechanism for this change is uncertain.

IT IS well known that the thyroid hormones, thyroxine (Th) and tri-iodothyronine (TRIT) have profound hypermetabolic effects on both intact organisms and isolated tissues. These hormones cause an increase in oxidative metabolism[1] of human erythrocytes. In addition, they have been shown to have effects on various enzyme systems.[2-4] Glock and McLean[5] showed significant increases in glucose-6-phosphate dehydrogenase (G-6-P. dehydrogenase) activity in the livers of thyroxine fed rats. In the current study, we have investigated levels of erythrocyte G-6-P. dehydrogenase activity with respect to thyroid hormone activity. Patients with hyperthyroidism, a patient with thyrotoxicosis factitia, and an euthyroid individual treated with thyroxine and tri-iodothyronine were investigated. A small series of patients with hypothyroidism was studied in addition. Finally, enzyme levels in the red cells of several animal species were measured in an attempt to correlate G-6-P. dehydrogenase with basal metabolism and red cell survival.

METHODS

Protein-bound iodine levels and studies with I^{131} were performed by standard techniques.[6, 7] The red cell uptake of tri-iodothyronine-I^{131} was performed by the method of Hamolsky and co-workers.[8]

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TABLE I

NO.	AGE	SEX	DIAGNOSIS	OPTICAL DENSITY CHANGE/ MIN.	HCT. RBC (%)	ENZYME UNITS/ 100 ML. RBC (N:117-186)	PROTEIN-BOUND IODINE (μg %) (N:3.0-7.0)	I^{131} UPTAKE (%) (N:15-45)	RBC TRIT-I^{131} UPTAKE (%) (N:11-18)	RETICULO-CYTES (%) (N:0.5-1.5)	PLASMA IRON TURNOVER (N:0.45 MG./KG./ DAY)
1.	34	F	Thyrotoxicosis factitia	.0402	90	432	17.2	1.0	34		
2.	30	M	Toxic goiter	.0258	88	283	9.9	78	45		
3.	26	M	Toxic goiter	.0206	86	231	11.8	75	22	1.0	
4.	36	F	Toxic goiter	.020	90	215	10.0	63	23		
5*a*.	25	M	Toxic goiter	.0196	92	206	9.3	54	26	0.8	
b.	25	M	2 mo. post thyroidectomy	.0102	60	164	4.4		12	0.6	
6.	30	F	Toxic goiter	.0270	94	278	8.7	58	25	0.8	
7*a*.	10	F	Toxic goiter	.0240	78	297	9.8	84	24	0.8	
b.	10	F	Toxic goiter; 3 mo. of propylthiouracil therapy	.0140	75	180	3.9		12	1.0	
8.	50	F	Toxic goiter	.0156	75	201	6.6	75	24.7		
9.	30	M	Toxic goiter	.0130	73	172	9.3	66	19	1.3	
10.	45	F	Toxic goiter	.0153	72	205	13.1	70	23	0.5	0.48
11.	42	F	Toxic goiter	.0102	43	229	9.5	69	20	0.4	0.22
12.	35	F	Toxic goiter	.0170	70	235	9.2	68	24	1.1	
13.	40	M	Potassium iodide therapy	.0114	82	134	18				
14.	33	M	Potassium iodide therapy	.0102	75	131	26				
15.	50	F	Hypothyroid	.0133	90	143	2.8	10	10		
16.	5	F	Hypothyroid with lingual thyroid	.0078	59	128	3.0	6			
17.	68	F	Hypothyroid	.0154	94	157	2.0	2	8.1		
18.	19	M	Athyrotic; off thyroid therapy 4 mo.	.0072	52	134	3.4	2.5	10.0		

G-6-P. dehydrogenase was measured by the method outlined by Zinkham.[9]

The Beckman DU spectrophotometer was used at a wavelength of 340 μ. The change in optical density per minute was measured between 5 and 12 minutes after addition of triphosphopyridine nucleotide (TPN) to 3.0 ml. of a buffered mixture of glucose-6-phosphate (G-6-P.) and hemolysate. An optical density change of 2.07 per minute per 3.0 ml. of reaction mixture represents the reduction of 1 micromole of TPN, and this is defined as 1 enzyme unit of G-6-P. dehydrogenase. The analysis was performed at a constant room temperature of 24° C.

In this laboratory, the level of G-6-P. dehydrogenase activity was found to average 137 ± 18.9 enzyme units per 100 ml. of packed red blood cells (range 117 to 186) in twenty-five normal children and adults. These values, although consistent, are lower than those reported by Zinkham.[9, 10] The reason for this is uncertain; however, our commercial source of TPN and G-6-P. was different from Zinkham's, and perhaps the discrepancy in values can be explained on the basis of reagent differences.

RESULTS

A. Patients with Hyperthyroidism.—Eleven patients with clinical and laboratory evidence of thyrotoxicosis were studied. High values were obtained for the serum protein-bound iodine, thyroidol I^{131} uptake and red blood cell uptake of TRIT-I^{131} in all cases as listed in Table I. None of these patients were anemic and reticulocyte counts, when performed, were normal. In ten of eleven cases, G-6-P. dehydrogenase activity was elevated. The average for the group was 232 ± 39 enzyme units per 100 ml. of red blood cells (range 172 to 297). This value is significantly different from the mean of the normal group ($P < 0.001$). One patient with severe thyrotoxicosis factitia had 432 enzyme units per 100 ml. of red blood cells, one of the highest levels of enzyme activity which we have observed. In two patients (Nos. 5 and 7) the determinations were repeated after return to an euthyroid state. The enzyme level in both instances had returned to within the normal range.

B. Thyroid Administration to an Euthyroid Individual.—A hematologically normal, euthyroid individual was given desiccated thyroid as outlined in Fig. 1. A significant increase in G-6-P. dehydrogenase activity was observed, which rapidly returned to the normal level when the medication was discontinued. Later tri-iodothyronine, 150 μg. per day, was given. After 48 hours, rapid rise of enzyme activity was again observed, but no change in reticulocytes occurred.

C. Effect of in Vitro Tri-iodothyronine (Table II).—Erythrocytes from a normal person were washed 3 times and suspended in buffered normal saline solution. They were then incubated for 1, 2½, and 24 hours with 1×10^{-5} moles of tri-iodothyronine per 1.0 ml. of red blood cells. This concentration of tri-iodothyronine has been shown to be active in increasing red cell oxidative metabolism in vitro.[1] No augmentation of enzyme activity was observed. After 24 hours of incubation, considerable decrease in G-6-P. dehydrogenase activity was observed, both in untreated blood and in that with added tri-iodothyronine. In addition, no effect was observed when tri-iodothyronine was added directly to hemolysate.

D. Elevation of Protein-Bound Iodine, Not on Basis of Hyperthyroidism.—G-6-P. dehydrogenase was measured in two patients with pulmonary disease who had been treated with potassium iodine. Although the protein-bound iodine was strikingly elevated, the enzyme activity was normal (Patients 13 and 14, Table I).

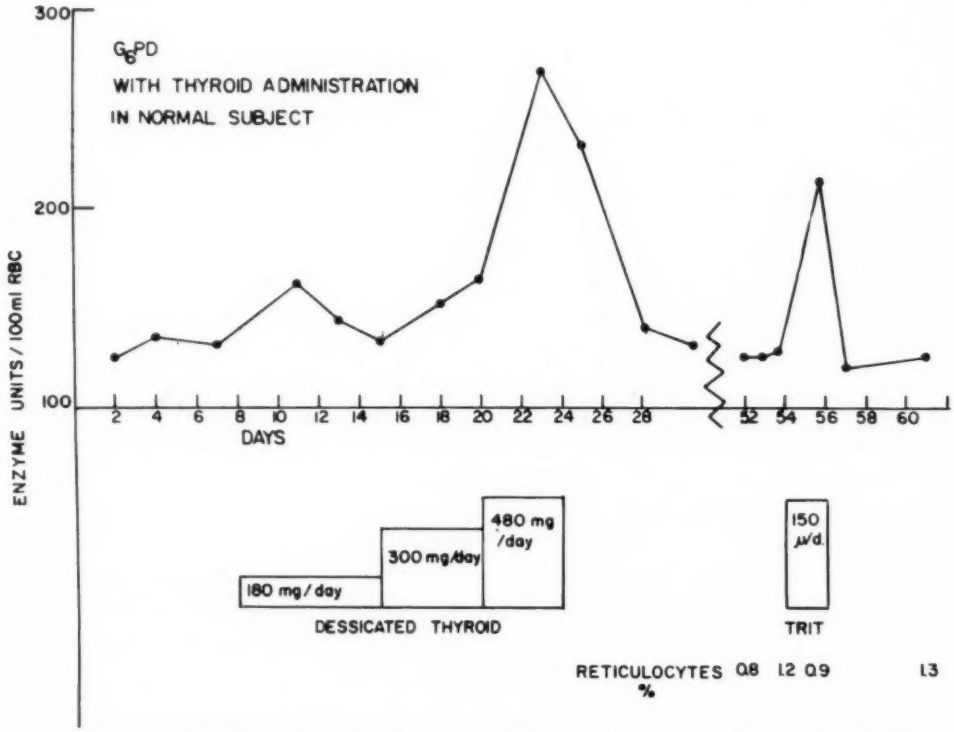

Fig. 1.—G-6-P. dehydrogenase activity correlated with administration of desiccated thyroid and tri-iodothyronine in an euthyroid hematologically normal subject.

E. Enzyme Levels in Hypothyroidism.—A small series of patients with hypothyroidism was studied (Patients 15 through 18 Table I). None were severely myxedematous. The G-6-P. dehydrogenase activity in the red cells of all of these individuals was in the normal range.

TABLE II. IN VITRO INCUBATION OF ERYTHROCYTES WITH TRI-IODOTHYRONINE (1×10^{-5} MOLES TRIT/ML. RBC)

	OPTICAL DENSITY CHANGE/MIN.	HCT.	ENZYME UNITS/ 100 ML. RBC
1. Control	10.6	70	146
2. Hemolysate + tri-iodothyronine	10.0	68	142
3. Incubation 1 hr.:			
a. Control	11.4	74	149
b. + tri-iodothyronine	10.6	62	155
4. Incubation 2½ hr.:			
a. Control	11.7	73	153
b. + tri-iodothyronine	10.2	69	143
5. Incubation 24 hr. at 37° C.:			
a. Control	3.4	64	51.5
b. + tri-iodothyronine	4.0	62	57.9

F. Enzyme Levels in Animals.—G-6-P. dehydrogenase was measured in several species of animals. In rats, rabbits, dogs, frogs, and turtles, the enzyme levels were within the normal range for humans. The enzyme activity in pooled red cells from 5 mice was 356 enzyme units per 100 ml.; however, the reticulocyte count of these red cells was 5.1 per cent. No consistent relation was observed between activity of this enzyme and the reported basal metabolic rates or red cell lifespan for the species[11] (Table III).

TABLE III

SPECIES	RBC* LIFESPAN (DAYS)	RETICULOCYTES (%)	G-6-P. DEHYDROGENASE ENZYME UNITS/100 ML. RBC	BASAL HEAT* PRODUCTION (CAL./Kg./24 HR.)
1. Mouse	22	5.1	356 (pooled cells from 7 animals)	171
2. Rat	58	1.9	153 (pooled cells from 3 animals)	83
3. Rabbit	55	1.8	156 (avg. of 2 animals)	45
4. Dog	100	1.1	170 (avg. of 2 animals)	35
5. Man	120	1.0	137	25
6. Frog			176 (pooled cells from 5 animals)	
7. Turtle	500		180 (avg. of 2 animals)	4.6

*Data summarized by Rodnan, Ebaugh, and Fox.[11]

DISCUSSION

A significant elevation of red cell G-6-P. dehydrogenase activity has been found in eleven of twelve cases of hyperthyroidism in humans. The mechanism of this increase is uncertain.

The most common conditions associated with elevated G-6-P. dehydrogenase activity are those with increased numbers of young red cells in the circulation. Young red cells, particularly reticulocytes, have increased levels of G-6-P. dehydrogenase activity.[12]

There is some evidence reported in the literature for a hemolytic process in thyrotoxicosis. McClellan and associates[13] reported shortened Cr^{51} half-times in two patients with thyrotoxicosis. However, Rodnan and Jensen[14] found a shortened Cr^{51} half-times in only one of seven cases. Heilmeyer[15] reported increased fecal urobilinogen in hyperthyroidism. Finally, Axelrod and Berman[16] described increased erythroid activity in the bone marrow of patients with hyperthyroidism.

However, none of our patients were anemic. Reticulocyte counts, when performed, were normal. In two patients with elevated enzyme levels, plasma iron turnovers, considered by Bothwell and collaborators[17] to be a sensitive index of increased erythropoiesis, were normal. Finally, a very rapid rise of enzyme activity was observed after administration of tri-iodothyronine to a normal person, although no change in the reticulocyte counts were observed. Therefore, we believe the reason for the observed elevation of erythrocyte G-6-P. dehydrogenase must be sought elsewhere.

Although there is danger in extrapolating from man to other species and vice versa, we found no consistent correlation between G-6-P. dehydrogenase activity and reported basal metabolism and red cell lifespans in different

animals. Thus, erythrocytes from the turtle, with its very low metabolic activity and markedly prolonged red cell lifespan, had enzyme levels in the high normal human range.

Other possible explanations to account for elevated G-6-P. dehydrogenase levels in hyperthyroid states could be either activation of enzyme systems or inhibition of enzyme antagonists. We were unable to augment enzyme activity by incubating tri-iodothyronine with red cells in vitro. It is of some interest that an in vivo excess of thyroid hormone should result in an increased enzyme activity, for some investigators have demonstrated inhibition rather than activation of various dehydrogenases by thyroxine.[18]

The observation of an elevated G-6-P. dehydrogenase level in hyperthyroid states is of theoretical interest; however, we believe that this finding will be of limited clinical value. There is a wide range of G-6-P. dehydrogenase values in normal individuals, and elevations are seen in hematologic conditions characterized by reticulocytosis. In addition, one of our hyperthyroid patients had high normal enzyme levels. Finally, the exact mechanism of the change is still uncertain. Nevertheless, we believe that more studies of this and other enzyme systems, which may be hormone responsive, should be undertaken to investigate both the mechanism and possible significance of these changes.

SUMMARY

Red cells from patients with hyperthyroidism showed significant elevation of G-6-P. dehydrogenase activity in eleven of twelve cases. In two instances, the enzyme level returned to normal after successful therapy of thyrotoxicosis. When thyroxine and tri-iodothyronine were administered to a normal individual, significant increases of G-6-P. dehydrogenase activity were observed. Two patients with high protein-bound iodine levels, on the basis of iodine therapy, had normal enzyme levels, as did four patients with hypothyroidism. In vitro incubation of thyroid hormones with erythrocytes did not augment G-6-P. dehydrogenase activity. We were unable to document the possibility of an increased rate of erythropoiesis as the basis for increased G-6-P. dehydrogenase levels. None of the patients were anemic. Reticulocyte counts were normal. Plasma iron turnover in two patients with high enzyme levels was normal. The mechanism of increased G-6-P. dehydrogenase activity in erythrocytes under the stimulus of thyroid hormone in vivo is still uncertain.

REFERENCES

1. Necheles, T., and Beutler, E.: The Effect of Tri-iodothyronine on the Oxidative Metabolism of Erythrocytes. I. Cellular Studies, J. Clin. Invest. **38:** 788, 1959.
2. Nishikawara, M. T.: Thyroid Control of Glucokinase and Glucose-6-Phosphatase Activity in Rat Intestinal Mucosa, Fed. Proc. **17:** 237, 1958.
3. Harary, I.: In Vivo Stimulation of Acyl Phosphatase by Thyroxine, Fed. Proc. **17:** 237, 1958.
4. Tipton, S. R., Zirk, M., and St. Amand, G. S.: Effect of Thyroxine on Mitochondrial Enzyme Activity in Rat Tissues, Fed. Proc. **17:** 174, 1958.
5. Glock, G. E., and McLean, P.: A Preliminary Investigation of the Hormonal Control of the Hexose Monophosphate Oxidative Pathway, Biochem. J. **61:** 390, 1955.
6. Spencer, R. P., King, E. R., Henkelmann, C. R., and Balbus, T. G.: A Discussion of Thyroid Parameters, Ann. Int. Med. **48:** 1046, 1958.

7. Barker, S. B.: Determination of Protein-Bound Iodine, J. Biol. Chem. **173**: 715, 1948.
8. Hamolsky, M. W., Stein, M., and Freedberg, A. S.: The Thyroid Hormone-Plasma Protein Complex in Man. II. A New in Vitro Study for "Uptake" of Labeled Hormonal Components by Human Erythrocytes, J. Clin. Endocrinol. **17**: 33, 1957.
9. Zinkham, W. H.: An in Vitro Abnormality of Glutathione Metabolism in Erythrocytes From Normal Newborns: Mechanism and Clinical Significance, Pediatrics **23**: 18, 1959.
10. Zinkham, W. H., and Lenhard, R. E.: Metabolic Abnormalities of Erythrocytes From Patients With Congenital Non-spherocytic Hemolytic Anemia, J. Pediat. **55**: 319, 1959.
11. Rodnan, G. P., Ebaugh, F. G., Jr., and Fox, M. R. S.: The Lifespan of the Red Blood Cell and the Red Blood Cell Volume in the Chicken, Pigeon and Duck, as Estimated by the Use of $Na_2Cr^{51}O_4$. With Observations on Red Cell Turnover Rate in the Mammal, Bird and Reptile, Blood **12**: 355, 1957.
12. Marks, P. A., and Gross, R. T.: Erythrocyte Glucose-6-Phosphate Dehydrogenase Deficiency: Evidence of Differences Between Negroes and Caucasians With Respect to This Genetically Determined Trait, J. Clin. Invest. **38**: 2253, 1959.
13. McClellan, J. E., Donegan, C., Thorup, O. A., and Leavell, B. S.: Survival Time of the Erythrocyte in Myxedema and Hyperthyroidism, J. LAB. & CLIN. MED. **51**: 91, 1958.
14. Rodnan, G. P., and Jensen, W. N.: A Study of Red Blood Cell Survival in Hypo- and Hyperthyroidism, Clin. Res. Proc. **5**: 8, 1957.
15. Heilmeyer, L.: Blutfarbstoffwechselstudien; Blutmauserung und Leberfunktion beim Morbus Basedow, Deutsches Arch. f. klin. Med. **171**: 515, 1931.
16. Axelrod, A. R., and Berman, L.: The Bone Marrow in Hyperthyroidism and Hypothyroidism, Blood **6**: 436, 1951.
17. Bothwell, T. H., Hurtado, A. V., Donohue, D. M., and Finch, C. A.: Erythrokinetics. IV. The Plasma Iron Turnover as a Measure of Erythropoiesis, Blood **12**: 409, 1957.
18. Wolff, J., and Wolff, E. C.: The Effect of Thyroxine on Isolated Dehydrogenases, Biochim. et biophys. acta **26**: 387, 1957.

REPRODUCIBILITY OF THE RADIOISOTOPE RENOGRAM

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In order to investigate the reproducibility of the radioisotope renogram, four normal individuals were subjected to repeated renograms using standardized conventional techniques. The curves obtained are analyzed and slopes, amplitudes, and duration values for the various phases are recorded. A statistical evaluation of the results is made which indicates that reproducibility is insufficient to permit quantitative evaluation of the renogram. Certain slope values and ratios of amplitudes show the best reproducibility of the values measured. Positioning of the detecting devices using acute caudal angulation is considered to be the most difficult variable to control and the one most responsible for the lack of reproducibility. Methods for overcoming this difficulty are discussed.

SINCE the introduction of the radioisotope renogram as a method for evaluating unilateral renal function, it has been generally assumed that this test has value only in a qualitative sense. No quantitative analysis of renogram curves has been successfully attempted because, it has been argued, the marked variability of the renogram precludes rational quantitation.[1, 2] However, the great potential value of a reliable precise method of studying unilateral renal function—especially a method as safe, painless, and easily performed as the radioisotope renogram—warrants a more intensive investigation of the possibility for quantitative analysis in this test.

As the first step in such an investigation the present report proposes to determine the reproducibility of the renogram in individuals with stable renal function. Analysis of the renogram curves would indicate whether quantitation is possible, and if so, which characteristics of the curves are most constant. These characteristics could then be investigated more thoroughly to establish measurable values for normal and abnormal renograms.

MATERIALS AND METHODS

Initially the variables involved in the performance of the renogram were evaluated. This was done to establish standard conditions for performing this study. The variables may be divided into two general categories as follows:

1. *Controllable Variables.*—Controllable variables are (a) dose of I^{131}-tagged Diodrast, (b) volume of fluid with which this is injected, (c) rate of injection, (d) state of hydration of the patient, (e) position of the patient, (f) position of the detecting devices, and (g) sensitivity of the detecting devices.

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2. *Noncontrollable Variables.*—Noncontrollable variables are (a) thickness of the tissue between the kidneys and the detecting devices, (b) size of the liver and its proximity to the right kidney, (c) intravascular fluid volume, and (d) physiologic variations of renal function.

In our Radioisotope Facility three scintillation measuring instruments are used for the renogram determination. One is used for each kidney and the third for monitoring general vascular activity. The levels of activity as "seen" by each scintillation detector are recorded as scale deflections on strip chart recorders.

Technically, each instrument contains as components:

1. Bell-Francis scintillation detector with flat field collimator. The detector has a 2 by 2 inch NaI (T1) crystal. The collimator subtends a solid angle of 30 degrees.

2. Bell-Francis medical spectrometer modified to give a one second time constant.

3. Varian potentiometer strip recorder utilizing rectangular co-ordinate recording system. This is modified by addition of a potentiometer to permit adjustment of the recorder scale deflection independently of the output of the spectrometer. The paper runs at a speed of 16 inches per hour.

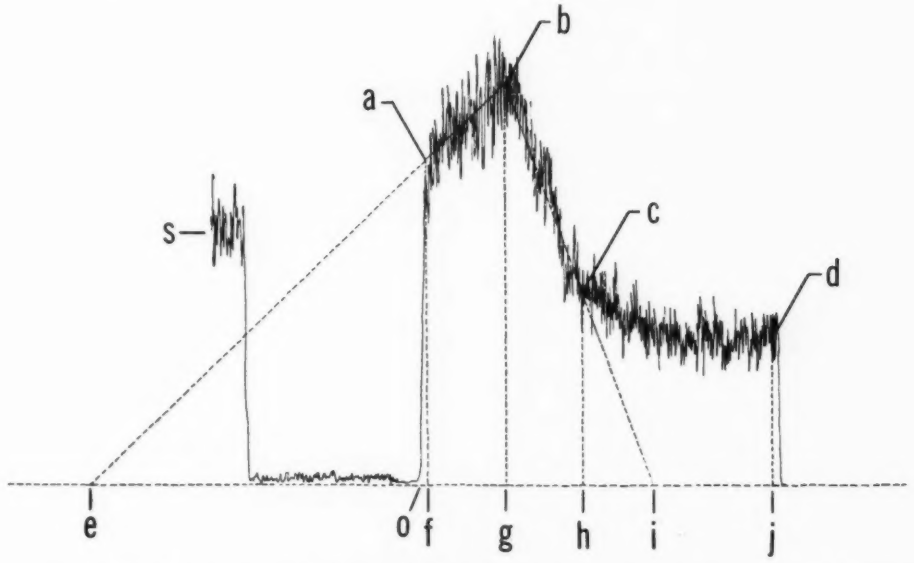


Fig. 1.—The measurements discussed in the text were made as illustrated. A typically normal renogram is shown in solid lines with amplitudes and slopes plotted on the curve in dotted lines. *S*, Standardization; *O*, beginning of injection; *fg*, duration of secretory (B) phase; *gh*, duration of excretory (C) phase; *af*, amplitude at end of vascular (A) phase; *bg*, amplitude at end of secretory (B) phase; *ch*, amplitude at end of excretory (C) phase; *dj*, amplitude at end of 15 minutes. 100/eg = slope of secretory (B) phase (per cent of *bg* per minute); 100/gi = slope of excretory (C) phase (per cent of *bg* per minute).

The analyzers are adjusted to measure the 364 Kev. photopeak of I^{131}. This provides discrimination against photons which might otherwise be counted, but which do not originate in the "cone of acceptance" of the detectors. Such scattered radiations come from many sources throughout the body, the one of chief concern being the liver.

In practice, the three channels are allowed to warm up sufficiently and then are adjusted for the 364 Kev. I^{131} peak. A source of I^{131} is placed in the center of a wood phantom approximately 5 inches in diameter. Each detector head is positioned so that its collimator touches the wood with the axis of the detector head in line with the source. Thus, each detector is the same distance from the source. The Varian recorder sensitivities are then adjusted so that they all have the same scale readings. One microcurie of I^{131} in the phantom gives a scale deflection of one half full scale.

TABLE I

	R. B. (MEAN VALUES FOR 5 RENOGRAMS ± THE AVERAGE DEVIATION)		D. B. (MEAN VALUES FOR 5 RENOGRAMS ± THE AVERAGE DEVIATION)		L. L. (MEAN VALUES FOR 5 RENOGRAMS ± THE AVERAGE DEVIATION)		T. W. (MEAN VALUES FOR 4 RENOGRAMS ± THE AVERAGE DEVIATION)	
	RIGHT KIDNEY	LEFT KIDNEY	RIGHT KIDNEY	LEFT KIDNEY	RIGHT KIDNEY	LEFT KIDNEY	RIGHT KIDNEY	LEFT KIDNEY
Amplitude (cm.) at end of vascular (A) phase	5.36 ± 1.0	5.22 ± 1.5	5.60 ± 0.7	4.34 ± 0.9	7.26 ± 0.6	6.16 ± 1.6	8.03 ± 0.3	8.72 ± 1.1
Amplitude (cm.) at end of secretory (B) phase	7.16 ± 1.5	6.56 ± 1.6	7.60 ± 0.8	5.40 ± 0.7	9.26 ± 0.7	7.24 ± 1.7	9.98 ± 0.3	10.72 ± 1.5
Amplitude (cm.) at end of excretory (C) phase	2.94 ± 0.6	2.16 ± 0.5	5.14 ± 1.3	2.30 ± 0.4	4.34 ± 1.3	3.38 ± 0.5	5.08 ± 0.6	4.60 ± 0.6
Amplitude 15 min. after injection	2.64 ± 0.6	1.94 ± 0.5	4.50 ± 1.4	1.72 ± 0.4	3.70 ± 0.6	2.84 ± 0.5	4.32 ± 0.5	4.12 ± 0.9
Slope of B phase	8.27 ± 0.8	7.99 ± 1.4	6.7 ± 2.1	10.68 ± 6.2	9.33 ± 2.4	6.06 ± 2.5	11.05 ± 2.2	10.55 ± 1.6
Slope of C phase	21.56 ± 1.7	24.2 ± 4.7	9.0 ± 5.4	13.77 ± 2.2	12.86 ± 3.3	11.30 ± 1.3	20.6 ± 3.1	22.72 ± 4.4
Duration (min.) of B phase	3.06 ± 0.4	2.78 ± 0.2	4.2 ± 0.9	2.46 ± 0.6	2.80 ± 0.5	3.20 ± 1.1	2.05 ± 0.4	2.12 ± 0.3
Duration (min.) of C phase	3.24 ± 0.7	3.1 ± 0.2	5.1 ± 1.9	4.24 ± 0.5	4.44 ± 0.3	4.76 ± 0.9	2.55 ± 0.4	2.78 ± 0.5
Ratio of amplitudes, B:A	1.33 ± .05	1.27 ± 0.6	1.36 ± .10	1.27 ± 0.12	1.28 ± .05	1.19 ± .04	1.27 ± .02	1.23 ± .03
Ratio of amplitudes, B:C	2.44 ± .29	3.06 ± .33	1.54 ± .23	2.35 ± 0.31	2.34 ± .55	2.13 ± .22	1.98 ± .32	2.33 ± .11

Incorporating the experience obtained in the performance of over one hundred renograms, standards were established for each of the controllable variables. Kidney position is determined by an upright x-ray film of the abdomen. The distances from the iliac crest to the upper pole of each kidney are measured on the x-ray film and marked on the patient's back. Each subject in a well-hydrated postprandial state is seated in a slightly forward leaning position.

Scintillation detectors are directed at the upper pole of each kidney, angulated 20 degrees medially and 50 degrees caudally with the perpendicular to the plane of the back. A third scintillation counter is directed at the muscle mass of the right upper arm to record general vascular activity. I^{131}-tagged Diodrast in the dose of 0.385 μc per kilogram body weight and diluted to a total volume of 4 ml. with normal saline is rapidly injected (within 3 seconds) into a left antecubital vein. The level of activity measured by each scintillation detector is then recorded for a 15 minute period.

A group of four healthy normotensive young men with no previous history of renal disease was studied. These subjects had normal values for blood urea nitrogen, urinalysis, and complete blood count. They were subjected to repeated renograms, from four to six in number. The renograms were performed under standard conditions at intervals of approximately one week. For each renogram a curve approximating the average activity measured was constructed on tracing paper placed over the renogram. This curve was divided into A (vascular), B (secretory), and C (excretory) phases according to the method of Winters (Fig. 1).[5, 6] Each tracing was then subjected to analysis, the following measurements being made: (a) amplitude in centimeters at the end of each phase, (b) amplitude at 15 minutes after injection, (c) duration of each phase, (d) slopes of the B and C phases, and (e) ratios of the amplitude at the end of phase B to those of A and C.

RESULTS

The results are presented in tabular and graphic form. Table I presents the average of each measured value together with the average deviation from

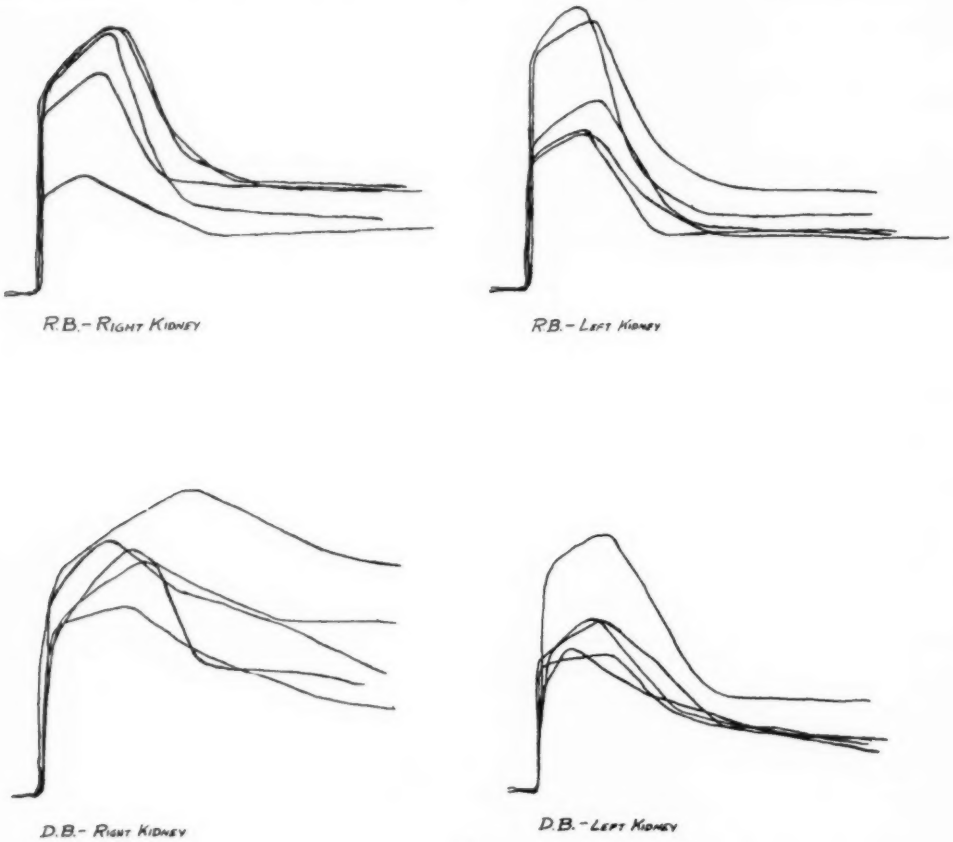
TABLE II

	OVER-ALL AVERAGE DEVIATION FROM THE MEAN (% OF MEAN)	CHOSEN LIMITS OF ACCEPTABLE DEVIATION FROM THE MEAN (%)	PERCENTAGE OF DETERMINATIONS WITH DEVIATIONS FROM THE MEAN EXCEEDING THE CHOSEN LIMITS (%)	INTERVAL WITHIN WHICH % OF DETERMINATIONS EXCEEDING THE CHOSEN LIMITS MAY BE EXPECTED TO LIE AT 90% CONFIDENCE LEVEL* (%)
Amplitude (cm.) at end of vascular (A) phase	16.4	± 20	34.2	24-46
Amplitude (cm.) at end of secretory (B) phase	14.7	± 20	34.2	24-46
Amplitude (cm.) at end of excretory (C) phase	19.3	± 20	39.5	29-51
Amplitude 15 min. after injection	21.3	± 20	39.5	29-51
Slope of B phase	27.4	± 40	28.9	19-40
Slope of C phase	21.9	± 40	15.7	8.5-26
Duration (min.) of B phase	19.8	± 20	34.2	24-46
Duration (min.) of C phase	17.1	± 20	34.2	24-46
Ratio of amplitudes, B:A	4.6	± 10	10.5	5-20
Ratio of amplitudes, B:C	13.2	± 20	21.1	12.5-32

*Figures obtained from "Tables of the Cumulative Binomial Probability Distribution" from the Harvard University Press.

the mean. Fig. 2 presents composites of renogram tracings for each kidney. Four curves comprise the composite for each kidney of subject T. W. and five for each kidney of the remaining subjects.

In order to evaluate these results it is first necessary to establish limits of acceptable deviation for each measured value. Ideally, these limits should be derived from a series of normal renograms compared to a series of abnormal renograms in a manner which would permit easy differentiation between the



A.

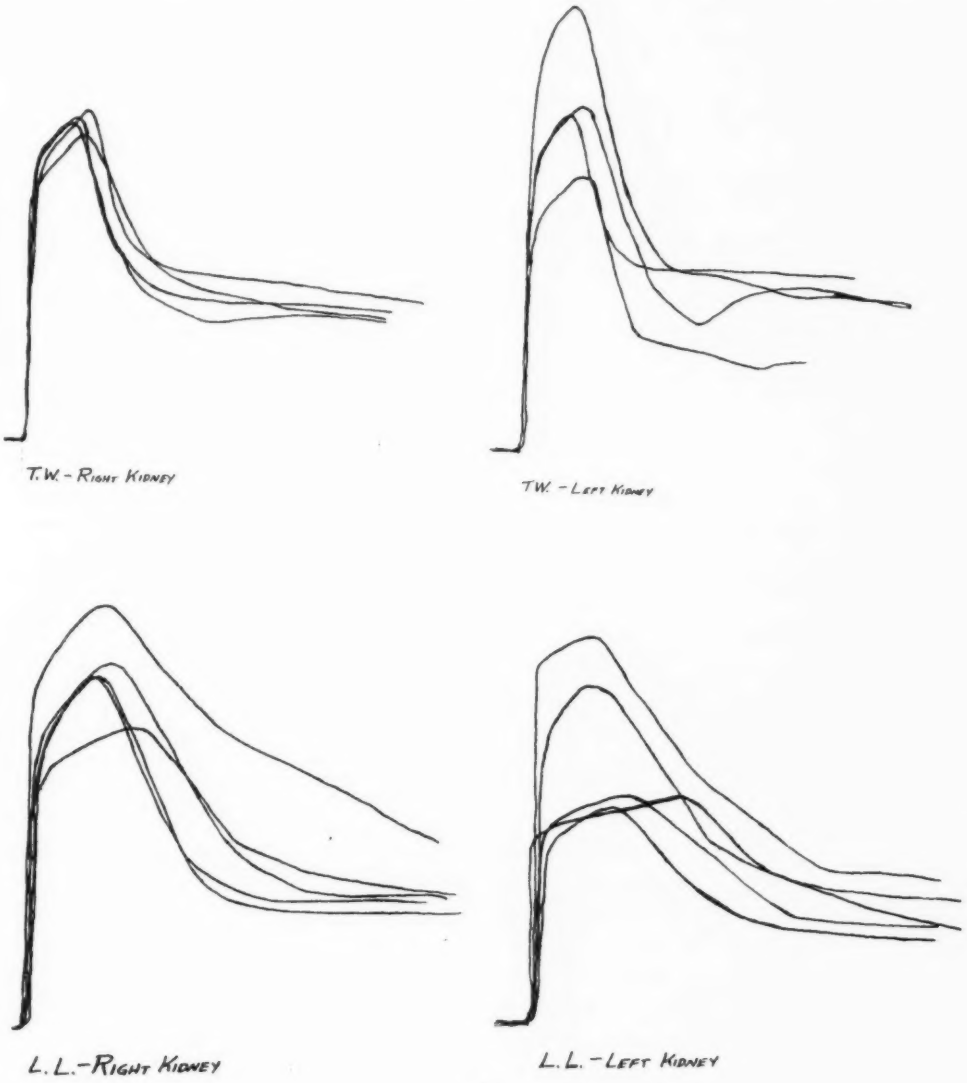
Fig. 2.—*A* and *B*, The composites of the renogram tracings for each of the kidneys of the four subjects, constructed as described in the text.

two groups. However, the existence of such series depends on the reproducibility of the renogram which is to be investigated in this article.

Therefore, in the absence of such information, limits of acceptable deviation were assigned by comparing a number of typically normal and abnormal renograms as performed in our laboratory.

The following deviations from mean values were taken as acceptable: amplitude A, 20 per cent; amplitude B, 20 per cent; amplitude C, 20 per cent; amplitude at 15 minutes, 20 per cent; B/A ratio, 10 per cent; B/C ratio, 20 per cent; slope AB, 40 per cent; slope BC, 40 per cent; duration of A phase, 20 per cent; duration of B phase, 20 per cent; duration of C phase, 20 per cent.

Column 1 of Table II gives the over-all average per cent deviation from the mean for each measured value. These were calculated by converting the average deviations from Table I to per cent deviations. The per cent deviations for each measured value were then averaged. Presented in Column 3 of



B.

Fig. 2*B*.—For legend see opposite page.

Table II is the percentage of determinations for all subjects with deviations from the mean exceeding the acceptable values as previously described.

Statistical Considerations.—The total number of determinations analyzed, considering each kidney as a separate unit, was 38; that is, ten determinations for each of three subjects and eight for the fourth. Therefore, for each of

the measured characteristics there are 38 values which either fall within or exceed the previously defined acceptable limits of deviation from the mean.

Column 3 in Table II lists the percentage of the determinations which exceed the assigned limits for each characteristic. In our study the limited number of renograms performed raises the question of whether the observed results accurately reflect the true results, i.e., those which would be obtained if a much larger series of renograms was performed. This can be statistically evaluated using the Cumulative Binomial Probability Distribution Tables.

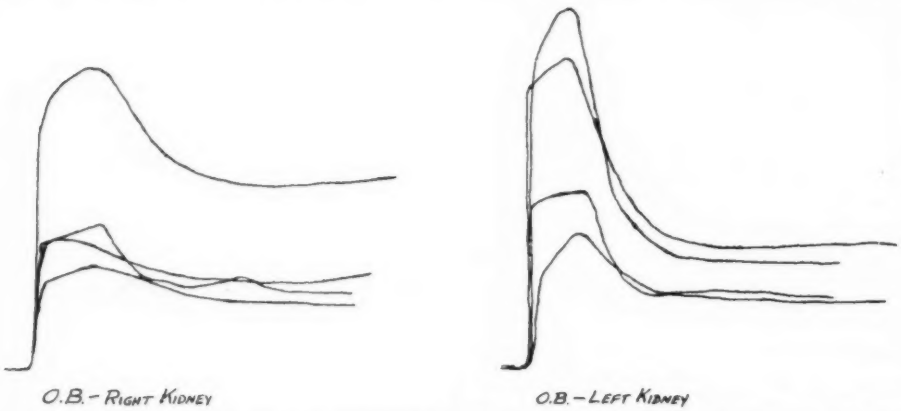


Fig. 3.—A composite of four renograms performed on an individual with marked rotoscoliosis in whom positioning of the detecting devices was difficult. Note the markedly varying results.

The confidence intervals for each of the figures in Column 3 of Table II are calculated at a 90 per cent confidence level and are presented in Column 4 of the same table. The smaller and larger figures represent the minimum and maximum percentages, respectively, of results exceeding the chosen limits of deviation from the mean which could be expected in a much more extensive series than the present one.

DISCUSSION

As may be seen by inspecting the data from the tables and the composite renogram tracings there is considerable variation in the curves obtained and, consequently, in the values measured. For most of these values, the deviations exceed the chosen limits of acceptability in a significant percentage of determinations. Considering even the most favorable possible situation, i.e., using the percentages at the lower end of the confidence intervals, the percentage of determinations exceeding the acceptable limits of deviation for most of the values considered is significant and probably prohibitive of quantitative evaluation.

The values which do show acceptable variations are amplitude ratios and slopes of the secretory and excretory phases. This would indicate that the principal difficulty involves factors which affect the absolute amplitudes of the curves. The single controllable variable which we have found to be most critical in this respect is the positioning of the scintillation detectors. Prior

to our establishing uniform positioning of both patient and detectors, it was not uncommon to produce entirely different appearing curves on renograms repeated on the same individual. A fifth subject upon whom four renograms were performed was excluded from this series because of extreme difficulty in positioning the scintillation detectors as a result of the subject's marked rotoscoliosis. A composite of his renogram tracings are presented in Fig. 3 which illustrates the marked variation introduced by inaccurate positioning.

With the standard conditions used, results have definitely improved over previous attempts but are still unsatisfactory for quantitative evaluation as previously discussed. Adjusting the scintillation detectors to a less acute caudal angle should improve reproducibility but would introduce hepatic interference caused by concentration of Diodrast in the liver.

Several changes in technique have been tried by others in an effort to circumvent this difficulty. The use of media other than Diodrast has tended to eliminate hepatic secretion. However, the mode of excretion of these other media are somewhat different and the characteristics of the curves are changed from those secured with Diodrast.[1, 4] At the present time reports appear to be most satisfactory with the use of Hypaque* tagged with I^{131}.[1]

The administration of carrier Diodrast to saturate the liver's avidity for the dye prior to the administration of the I^{131}-tagged Diodrast is reported.[2] There is some disagreement on the value of this procedure. The present writers have had no experience with this technique.

It is not intended that any of the preceding discussion detract from the value of the radioisotope renogram as a qualitative test. As Winter has suggested, the test is of considerable value in screening hypertensive individuals for unilateral renal disease. However, we wish to stress that an attempt to establish a range of normal values for radioisotope renograms or even limits of difference between the two kidneys where the tracings are obtained simultaneously would be premature using the present techniques with their inherent variability.

SUMMARY AND CONCLUSIONS

1. Four patients with ostensibly normal renal function were subjected to multiple repeated renograms under standard conditions. Reproducibility of the curves was investigated. Quantitative and statistical analyses of the results are presented.

2. Despite carefully controlled test conditions, variations which may preclude quantitative evaluation may be expected with radioisotope renograms using techniques described here.

3. *Slopes* and *ratios of amplitudes* show the greatest reproducibility of the values investigated.

4. Positioning of the patient and of the scintillation detecting devices (using extreme caudal angulation) are the variables most difficult to control. This contributes significantly to the lack of reproducibility.

*Subsequent to the submission of this article for publication, the authors and others have been evaluating the use of iodohippuric acid tagged with I^{131} for renal function studies. This appears at the present time to be the most satisfactory of all of the agents used to date for renal function studies.

We are indebted to Virginia Hanly and Thomas Willke for assistance in the statistical evaluation of our material.

REFERENCES

1. Winter, C. C., Maxwell, M. H., Rockney, R. E., and Kleeman, C. R.: Results of Radioisotope Renogram and Comparison With Other Kidney Tests, J. Urol. **82**: 674, 1959.
2. Serratto, M., Grayhack, J. T., and Earle, D. P.: A Clinical Evaluation of the Iodopyracet (Diodrast) Renogram, A. M. A. Arch. Int. Med. **103**: 851, 1959.
3. Winter, C. C.: Unilateral Renal Disease and Hypertension, Use of the Radioactive Diodrast Renogram as a Screening Test, J. Urol. **79**: 573, 1958.
4. Winter, C. C., and Taplin, G. V.: A Clinical Comparison and Analysis of Radioactive Diodrast, Hypaque, Miokon and Urokon Renograms as Tests of Kidney Function, J. Urol. **79**: 573, 1958.
5. Taplin, G. V., Meredith, O. M., Jr., Kade, H., and Winter, C. C.: The Radioisotope Renogram, J. LAB. & CLIN. MED. **48**: 886, 1956.
6. Taplin, G. V., Meredith, O. M., Kade, H., and Winter, C. C.: The Radioisotope Renogram, Atomic Energy Project Report, May 2, 1956.

LIVER ARGINASE ACTIVITY IN PATIENTS WITH LIVER CIRRHOSIS AND IN PATIENTS IN ENDOGENOUS HEPATIC COMA

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The high blood ammonia observed in some patients in hepatic coma, the elevation of serum arginase in these patients, and the lack of success of L-arginine in the treatment of this condition have induced us to investigate liver arginase activity in hepatic failure. Hepatic arginase activity was assayed in liver homogenates by the method of Archibald and Van Slyke in ten so-called normal controls, six patients with cirrhosis of the liver, and twelve patients in endogenous hepatic coma. Hepatic arginase activity in the control group averaged 9.5 ± 3.7 μM urea per milligram; in the patients with cirrhosis the mean was 7.4 ± 1.68 and it was found significantly diminished in the group in hepatic coma with a mean of 2.5 ± 1.16 ($p < 0.001$). Blood ammonia and serum arginase were determined in the patients in coma and found elevated. Blood urea was low or normal.

A HIGH ammonia content has been found in the blood of patients with cirrhosis of the liver especially in those who have lapsed into hepatic coma. This finding suggests that blood ammonia may be related to the neuropsychiatric signs of hepatic coma.[1-3] The elevated blood ammonia observed in patients with diffuse liver disease has been attributed to the numerous intrahepatic and extrahepatic portacaval shunts which prevent detoxification of ammonia and possibly other toxic products by the liver cells. Ammonia may also be inefficiently metabolized because of severe hepatocellular failure. The first of these mechanisms has been extensively studied in man and in the laboratory animal[4]; little attention, however, has been paid to the study of ammonia metabolism in hepatic failure.[5] The ammonia is normally removed rapidly by the liver which is the only organ capable of forming urea. Urea synthesis was first described as a cyclic process by Krebs and Henseleit.[6] In this cycle they found three amino acids involved, ornithine, citruline, and arginine; the enzyme, arginase, accomplished the final hydrolysis of arginine to ornithine and urea. A description of this cycle and the enzymatic reactions involved can be found in recent articles by Brown[7] and Naharian and Harper.[8]

The facts that L-arginine has been employed without success in the treatment of hepatic coma by the majority of those who tried this amino acid and our finding of elevated serum arginase activity in patients with cirrhosis of the liver have induced us to think that liver arginase may be diminished in severe hepatic failure.[9-11] Therefore, we have investigated liver arginase activity in patients who died in irreversible hepatic coma, in liver samples of cirrhotic patients not in coma, and in so-called normal livers.

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MATERIALS AND METHODS

Hepatic arginase activity was assayed in liver samples of the following patients: (1) a control group which consisted of six patients who underwent laparotomy because of non-hepatic illness and four deceased patients on whom autopsies were performed. The autopsy group was made up of the following patients: one with acute nephrosis (with renal failure and a very high blood urea nitrogen), one with heart failure due to mitral insufficiency and stenosis, one with cancer of the gall bladder and hydronephrosis in whom no hepatic involvement was found at autopsy, and one with cancer of the uterus without liver metastases at autopsy (Table I). (2) The cirrhotic group consisted of ten patients with cirrhosis of the liver who died in hepatic coma and six who were not in coma in whom the liver samples were obtained, three at laparotomy and three at autopsy. Two patients with fulminant hepatitis who died in hepatic coma were also studied (Tables II and III).

The liver samples obtained at autopsy and kept in the freezer at −4° C. were analyzed within 24 hours of the death of the patients. The same time elapsed between obtaining the surgical samples and their analysis. A liver homogenate was made according to the method of Potter and Elvehjem[12] as modified by Wilbur and Skeen.[13] In this homogenate protein was determined by the biuret method[14] and arginase by the method of Archibald and Van Slyke[15] as modified by Cruz Coke and associates.[6] Five tenths milliliter cobaltous chloride 0.0015M was used as an activator. Arginase activity was expressed in micromoles of urea per milligram of protein. In the patients with liver disease a clinical history was recorded and the following laboratory tests were performed: serum bilirubin,[16] cholesterol,[17] flocculation tests,[18, 19] prothrombin concentration,[20] serum glutamic oxalacetic transaminase,[21] and uremia by hypobromite method. In five patients arterial blood ammonia was assayed[3, 22] and in nine patients serum arginase activity was determined.[11]

RESULTS

Hepatic arginase activity in the control group averaged 9.5 ± 3.7 μM urea per milligram of protein (Table I).

TABLE I. HEPATIC ARGINASE ACTIVITY IN CONTROL GROUP

CASE	SOURCE OF SAMPLE	HEPATIC ARGINASE (μM UREA/MG. PROTEIN)
1	Surgical biopsy	6.2
2	Surgical biopsy	10.7
3	Surgical biopsy	5.0
4	Surgical biopsy	13.8
5	Surgical biopsy	4.8
6	Surgical biopsy	11.3
7	Cancer of uterus*	9.7
8	Heart failure*	10.4
9	Acute renal failure*	8.1
10	Gall bladder carcinoma*	16.7
	Mean	9.5
	Standard deviation	3.7
	Error of the mean	1.18

*Autopsy.

Table II summarizes the clinical and laboratory data of the twelve patients who died in hepatic coma. As can be seen in Table II the twelve patients who died in coma had clinical and laboratory signs of advanced hepatic failure. In these patients no exogenous factors responsible for the coma such as exaggerated ingestion of proteins, gastrointestinal bleeding, or the ingestion of comatogenic drugs could be demonstrated. The neuropsychic symptoms observed in these patients were therefore considered as related to the liver failure.

Table III shows the weight of the liver and the values of hepatic arginase, uremia, arterial blood ammonia, and serum arginase activity of the patients in hepatic coma. Hepatic arginase activity averaged in this group 2.5 ± 1.16 μM urea per milligram of protein. The weight of the liver was below normal in the majority of these cases; in those patients whose livers weighed more than normal, important areas of necrosis could be demonstrated by histopathologic examination.

In the patients in coma in whom blood ammonia was assayed this was elevated, ranging between 1.88 and 2.6 μg ammonia nitrogen (NH_3-N) per milliliter of blood (normal values in our laboratory up to 1 μg NH_3-N per milliliter of blood). Blood urea was normal or only slightly elevated in these patients.

Table IV describes the clinical, laboratory, and hepatic arginase data in the patients studied who were not in hepatic coma. The mean hepatic arginase activity in this group was 7.4 ± 1.68 μM urea per milligram of protein.

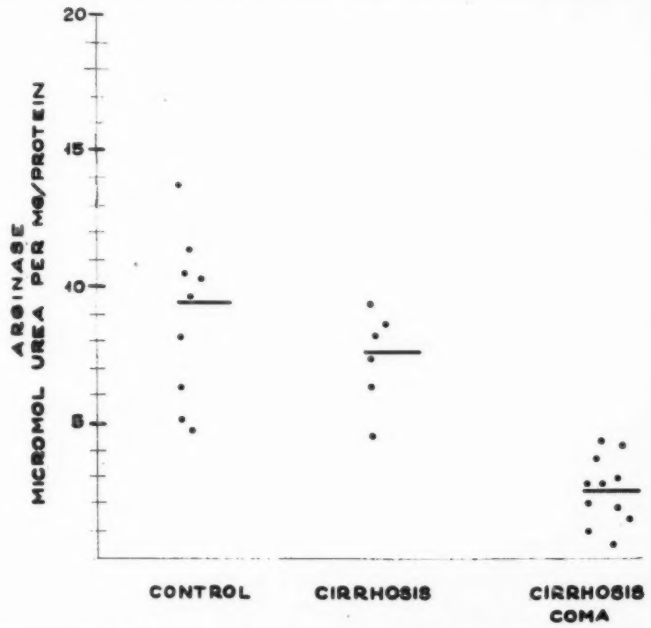

Fig. 1.—Summarizes the hepatic arginase activity results in the cases studied, each dot represents one case and the lines represent the averages.

In Fig. 1 hepatic arginase activity of the control group and the groups of patients in hepatic coma and without coma are compared. It can be seen easily that the means of the control group and the group in coma are clearly different, and a small difference can be appreciated between the control group and the group of patients who did not have very advanced liver disease and who were not in coma. The difference of means between the control group and the group of patients in hepatic coma was statistically significant ("t" of Student 5.7 and $p < 0.001$). The difference of means between the control subjects and the group of patients with cirrhosis not in coma was not statistically significant ($p > 0.05$).

TABLE II. CLINICAL AND BIOCHEMICAL DATA IN 12 CASES OF HEPATIC COMA STUDIED

CASE	COMA	ASCITES (MG. %)	TOTAL BILIRUBIN (MG. %)	1 MIN. DIRECT BILIRUBIN (MG. %)	CHOLESTEROL (MG. %)	CEPHALIN CHOLESTEROL FLOCCULATION TEST	THYMOL TURBIDITY (MACLAGAN UNITS)	PROTHROMBIN CONCENTRATION (%)	S-GOT (KARMEN UNITS)	DIAGNOSIS
1	++	Yes	9.7	3.07	139.7	+++	7.7	0	52.5	Chronic alcoholism Septal cirrhosis
2	++++	Yes	1.6	0.53	205	+++	16	40	60	Chronic alcoholism Septal cirrhosis
3	++++	Yes	2.3	0.82	94	+++	7.2	21	83.5	Chronic alcoholism Septal cirrhosis
4	+	Yes	2.0	0.62	145	+++	17	38	97.5	Postnecrotic cirrhosis
5	++++	No	17.3	0.62	142.5	++++	6.2	11	1,312.5	Fulminant hepatitis
6	++++	Yes	3.6	1.3	251	+++	10.2	21	65	Chronic alcoholism Septal cirrhosis
7	++++	Yes	26.7	11.0	140	++++	15.4	5	295	Fulminant hepatitis
8	++++	Yes	6.6	2.6	168.1	++++	11.8	27	60	Chronic alcoholism Septal cirrhosis
9	++++	No	18.2	7.9	97	++++	8.3	20	92.5	Hepatitis Subacute necrosis
10	+	Yes	10.6	4.8	230	+++	16.8	14	75.2	Chronic alcoholism Septal cirrhosis
11	+++	No	10.2	5.1	177	+++		45	80	Postnecrotic cirrhosis
12	++	Yes	2.8	1.05	125	+++	7.8	21	55	Chronic alcoholism Septal cirrhosis

TABLE III. ARTERIAL BLOOD AMMONIA, BLOOD SERUM ARGINASE, WEIGHT OF LIVER, AND HEPATIC ARGINASE IN PATIENTS IN HEPATIC COMA

CASE	NH_3-N (γ/ML. BLOOD)	UREMIA (MG. %)	SERUM ARGINASE (μM UREA/ML. SERUM)	LIVER WEIGHT (GM.)	HEPATIC ARGINASE (μM UREA/MG. PROTEIN)
1	1.88	40	—	1,080	2.03
2	—	20	4.05	1,200	3.2
3	2.1	20	—	1,210	1.0
4	—	—	—	1,400	3.8
5	—	40	1.32	820*	2.8
6	2.4	30	0	—	4.4
7	—	25	2.7	820*	2.9
8	2.6	23	3.4	1,160	2.8
9	—	60	0	1,120*	2.0
10	—	65	3.6	2,460*	4.1
11	—	20	5.6	815	0.4
12	—	110	0.99	1,520*	1.7
				Mean	2.5
				Standard deviation	1.16
				Error of the mean	0.33

*Liver necrosis.

DISCUSSION

Our results show, in the conditions in which the study was carried on, a marked diminution of hepatic arginase activity in patients who died in hepatic coma caused by advanced liver failure. These results, obtained 24 hours after death, represent to our belief the arginase activity present in the livers before death, because it has been demonstrated that this enzyme is very stable even at room temperature.[23] Successive analyses done by us in the same sample 3, 6, 12, and 24 hours after death showed no changes in the enzymatic activity in spite of the time lapsed.

The basis of reference for comparison of arginase activity between a normal and a cirrhotic liver constitutes an unresolved problem because in the cirrhotic liver a variable portion of the parenchyma is replaced by fibrous tissue. As collagen tissue is comparatively scanty in the nuclei, it has been suggested that enzyme activity in these cases should be expressed in relation to desoxyribonucleic acid. In this way a better representation of the active parenchyma could be obtained. However, this method does not resolve the problem because of the polyploid character of the liver cells, especially in the case of cirrhosis. Also the inflammatory infiltration so common in this disease[24] makes this solution uncertain.

Our findings of low hepatic arginase activity in two patients in coma caused by fulminant hepatitis and the normal values of hepatic arginase activity recorded in patients with cirrhosis who were not in coma induced us to think that the difference observed between the control group and the cirrhotic patients in hepatic coma cannot be attributed to the infiltration of collagen tissue in the latter group. The results reported by Coronado[25] in a miscellaneous group of cirrhotic patients who died from different causes such as drowning, cranial injury, etc., show much higher values of hepatic arginase than we

TABLE IV. CLINICAL AND BIOCHEMICAL DATA IN SIX PATIENTS WITH CIRRHOSIS WITHOUT COMA

CASE	ASCITES	TOTAL BILIRUBIN (MG. %)	DIRECT BILIRUBIN (MG. %)	CHOLESTEROL (MG. %)	CEPHALIN CHOLESTEROL FLOCCULATION TEST	THYMOL TURBIDITY (MACLAGAN UNITS)	PROTHROMBIN CONCENTRATION (%)	S-GOT (KARMEN UNITS)	HEPATIC ARGINASE	DIAGNOSIS
13	Yes	0.53	0.09	128	++	3.7	80	19.5	7.2	Alcoholic
14	Yes	1.4	0.57	116.8	+++	2.6	80	74	6.4	Alcoholic
15	—	0.4	0	188.3	+++	11	90		4.7	Alcoholic
16	Yes	2.8	1.1	180	+++	9.1	28	114	9.3	Alcoholic
17	No	0.4	0	194	+	3	50		8.1	Alcoholic
18	No	18.2	7.8	285	+++	8	80		8.7	Secondary biliary

have recorded in the patients in coma; this supports our belief that a real difference in arginase activity exists between normal livers and livers of patients with advanced cirrhosis in hepatic coma.

The low hepatic arginase activity observed in the patients studied by us is possibly explained as a failure of these seriously damaged livers to synthesize proteins, and perhaps also to the liberation into the blood stream of this enzyme by the injured cell.[11] Although our results show a clear-cut diminution of hepatic arginase activity in livers with advanced hepatocellular failure we do not yet have experimental facts to assume that this deficit will cause a blockage in the synthesis of urea.

Arginine administration to patients in hepatic coma has been reported to lower blood ammonia and raise blood urea.[8] However, Bessman[26] and Fahey[9] have found no effect of arginine on elevated blood ammonia in patients in coma caused by severe liver failure. These latter experiences and the fact that our patients in coma had a normal or a low blood urea suggest that at least in some cases, in advanced hepatic failure, a low hepatic arginase may explain the inefficiency of arginine to modify a high ammoniemia.

SUMMARY

1. Hepatic arginase activity was studied in so-called normal individuals, in patients with cirrhosis of the liver, and in patients in endogenous hepatic coma.

2. Hepatic arginase activity in the control group averaged 9.5 ± 3.5 μM urea per milligram of protein.

3. The group of cirrhotic patients not in coma had a mean hepatic arginase of 7.4 ± 1.68 μM urea per milligram of protein.

4. Hepatic arginase activity in the group in hepatic coma was low with an average of 2.5 ± 1.16 μM ($p < 0.001$).

REFERENCES

1. McDermott, W. V., Jr.: Metabolism and Toxicity of Ammonia, New England J. Med. **28**: 1076, 1957.
2. McDermott, W. V., Jr., and Adams, R. R.: Episodic Stupor Associated With an Eck Fistula in the Human With Particular Reference to the Metabolism of Ammonia, J. Clin. Invest. **33**: 1, 1954.
3. Bessman, S. P., and Bessman, A. N.: Cerebral and Peripheral Uptake of Ammonia in Liver Disease With a Hypothesis for the Mechanism of Hepatic Coma, J. Clin. Invest. **34**: 622, 1955.
4. Summerskill, W. H. J., Davidson, E. A., Sherlock, S., and Steiner, R. E.: Neuropsychiatric Syndrome Associated With Hepatic Cirrhosis and Extensive Portal Collateral Circulation, Quart. J. Med. **25**: 245, 1956.
5. Pearl, D. C., and McDermott, W. V., Jr.: A Vulnerable and Rate-Limiting Step in Urea Synthesis in Patients With Hiperammonaemia, Proc. Soc. Exper. Biol. & Med. **97**: 440, 1958.
6. Cabello, S.: La Arginasa Hepática, Santiago, 1955, Universidad de Chile.
7. Brown, G. W., and Cohen, P. P.: Comparative Biochemistry of Urea Synthesis, J. Biol. Chem. **234**: 1769, 1959.
8. Naharian, J. S., and Harper, H. A.: A Clinical Study on the Effect of Arginine on Blood Ammonia, Am. J. Med. **21**: 832, 1956.
9. Fahey, J. F., Nathans, D., and Rairich, D.: Effects of L-Arginine on Elevated Blood Ammonia Levels in Man, Am. J. Med. **23**: 860, 1957.
10. Fazekas, J. F., Ticktin, H. E., and Shea, J. G.: Effects of L-Arginine on Hepatic Encephalopathy, Am. J. M. Sc. **234**: 463, 1957.

11. Ugarte, G., Pino, M. E., Peirano, P., and Marusić, E.: Serum Arginase Activity in Subjects With Hepatocellular Damage, J. LAB. & CLIN. MED. **55**: 522, 1960.
12. Potter, V. R., and Elvehjem, C. A.: A Modified Method for the Study of Tissue Oxidation, J. Biol. Chem. **140**: 495, 1936.
13. Wilbur, K. M., and Skeen, M. V.: Sample Tissue Homogenizer, Science **111**: 304, 1950.
14. Kingsley, G. R.: The Direct Biuret Method for the Determination of Serum Proteins, as Applied to Photoelectric and Visual Colorimetry, J. LAB. & CLIN. MED. **27**: 840. 1942.
15. Van Slyke, D. D., and Archibald, R. M.: Gasometric and Photometric Measurement of Arginase Activity, J. Biol. Chem. **165**: 293, 1946.
16. Ducci, H., and Watson, C. S.: The Quantitative Determination of the Serum Bilirubin With Special Reference to the Prompt-Reacting and the Chloroform-Soluble Types, J. LAB. & CLIN. MED. **30**: 293, 1945.
17. Bloor, W. R.: Determination of Cholesterol in Blood, J. Biol. Chem. **24**: 227, 1916.
18. Neefe, J. R., and Reinhold, S. G.: Photosensitivity as a Cause of Falsely Positive Cephalin Cholesterol Flocculation Test, Science **100**: 83, 1944.
19. Ducci, H.: The Thymol Test of Maclagan, J. LAB. & CLIN. MED. **32**: 1266, 1947.
20. Quick, A. S.: The Physiology and Pathology of Hemostasis, Philadelphia, 1951, Lea & Febiger.
21. Sall, T., Richards, H. K., Harrison, E., and Myerson, R. F.: A Colorimetric Procedure for the Determination of Serum Glutamic Oxalacetic Transaminase, J. LAB. & CLIN. MED. **50**: 297, 1957.
22. Bessman, S. P.: Personal Communication.
23. Greenberg, D. M.: Arginase, *in* Summer, J. B., and Myrbäck, K., editors: The Enzymes, New York, 1951, Academic Press, Inc., vol. 1, p. 893.
24. Waterlow, S. C., and Patrick, S. J.: Enzyme Activity in Fatty Livers in Human Infants, Ann. New York Acad. Sc. **57**: 750, 1954.
25. Coronado, A.: Determinación de la actividad arginásica del hígado humano normal y en algunas condiciones patológicas, Tesis, 1956, Universidad de Chile, Facultad de Medicina.
26. Bessman, S. P., Shear, S., and Fitsgerald, S.: Effects of Arginine on the Removal of Ammonia From the Blood in Normal and Cirrhotic Patients, New England J. Med. **256**: 941, 1957.

BLOOD SACCHAROIDS IN DIABETES MELLITUS

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Blood saccharoid levels of twenty nondiabetic subjects (80 samples) and of thirty-five diabetic patients (140 samples) were determined as the difference between copper reduction and glucose oxidase methods. Mean values plus or minus the standard deviations for the normal and diabetic groups were 6.0 mg. per cent ± 4.0 and 21.0 mg. per cent ± 10.6, respectively. No significant difference was found between capillary and venous blood for either group. No changes were observed in the saccharoid levels of either diabetic or nondiabetic individuals during the modified glucose-insulin tolerance test or during the four hour interval after a single oral dose of 1 Gm. of tolbutamide. Elevation of the saccharoid level of the diabetic patients was not related to blood glucose values or to insulin sensitivity. By means of paper chromatographic techniques an organic acid was found in increased amounts in barium-zinc filtrates from diabetic blood which had the same RF value as glucuronic acid. It is suggested that in some diabetic patients alternate pathways of carbohydrate metabolism might result in increased production of glucuronic acid or other saccharoids.

THE term "saccharoids" was first suggested by Benedict[1] as a means of designating the entire group of substances in blood filtrates which are copper reducing but nonfermentable, i.e., nonglucose reducing compounds. Since the amount of saccharoids will vary considerably with different kinds of blood filtrates, the term "saccharoids" is defined in this article in a more restricted sense as the difference between glucose values obtained on zinc filtrates with the Nelson-Somogyi copper reduction method[2] and those obtained with the glucose oxidase procedure of Saifer and Gerstenfeld.[3]

In a recent publication from this laboratory,[3] it was reported that 2 to 15 mg. per cent of saccharoids were present in the blood of normal individuals. While some diabetic patients also showed normal saccharoid levels, many had saccharoid values greater than 20 mg. per cent. Similarly, Cawley and collaborators[4] reported an elevation of saccharoid levels in diabetic patients.

In the present investigation these earlier findings of elevated blood saccharoid levels in some diabetic patients have been confirmed. We have also compared the saccharoid levels of arterial and venous blood from normal and diabetic individuals, and studied the changes which occurred during the modified glucose-insulin tolerance test[5, 6] and after the administration of a single

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oral dose of tolbutamide. An attempt was made to identify the compounds responsible for the elevated saccharoid values in the diabetic individuals.

MATERIALS AND METHODS

Comparison of Saccharoid Values in Venous and Capillary Blood.—Capillary (fingertip) and venous bloods were drawn simultaneously from six diabetic patients and the glucose content of the respective laked whole blood samples were determined on the same barium-zinc filtrates with both the Nelson-Somogyi (N.S.)[2] and glucose oxidase (G.O.)[3] methods. Repeat determinations were then performed at 3 to 4 day intervals on the same six patients for a total of 5 determinations on each. The average values obtained for each run of the group of six diabetic patients are given in Table I. They show that about the same values are obtained for venous, as compared to capillary, blood, with both the Nelson-Somogyi and glucose oxidase methods. It should be noted that two of these six diabetic patients constantly exhibited saccharoid levels below 20 mg. per cent during the 2 week period while the tests were being run. The other four patients showed wider fluctuations of their saccharoid levels, but these were generally in excess of 20 mg. per cent. A typical set of results for a patient (No. 1) showing normal saccharoid values and another set showing elevated fluctuating values (Patient No. 6) are given at the bottom of Table I.

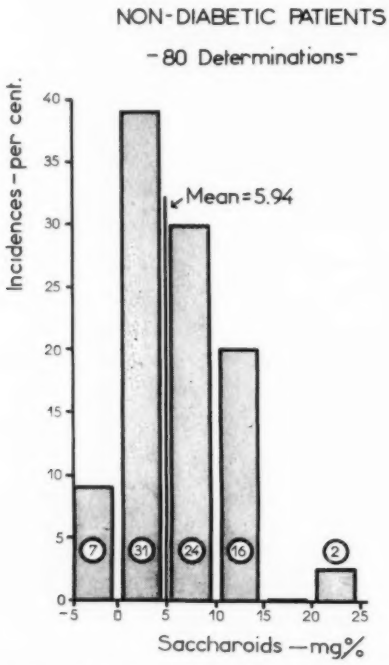

Fig. 1.—Distribution of saccharoid values obtained for a group of twenty nondiabetic patients (80 samples).

Saccharoid Levels of Nondiabetic Patients.—Saccharoid levels were determined simultaneously on both venous and capillary blood in a group of twenty nondiabetic patients on each of two occasions. The data obtained for the group represent 80 separate blood samples, each of which was run in duplicate. The saccharoid distribution for these individuals is plotted in Fig. 1. These data show that about 98 per cent of the saccharoid values are below 15 mg. per cent. The values range from −3 to +22 mg. per cent with a mean of 6.0 mg. per cent and a standard deviation of ±4.0 mg. per cent.

Saccharoid Levels of Diabetic Patients.—Saccharoid levels were determined simultaneously on capillary and venous blood on each of two occasions in a group of thirty-five diabetic patients, a total of 140 samples.

The saccharoid distribution for this group of diabetics is shown in Fig. 2. A mean value of 21 mg. per cent was obtained for the saccharoid level of the diabetic patients; this is 15 mg. per cent higher than that of the nondiabetic group. The range of saccharoid values for the diabetic group was from 0 to 65 mg. per cent, and 92 out of the 140 samples (or 65 per cent) fell above 15 mg. per cent, which is the accepted upper limit of the normal range.

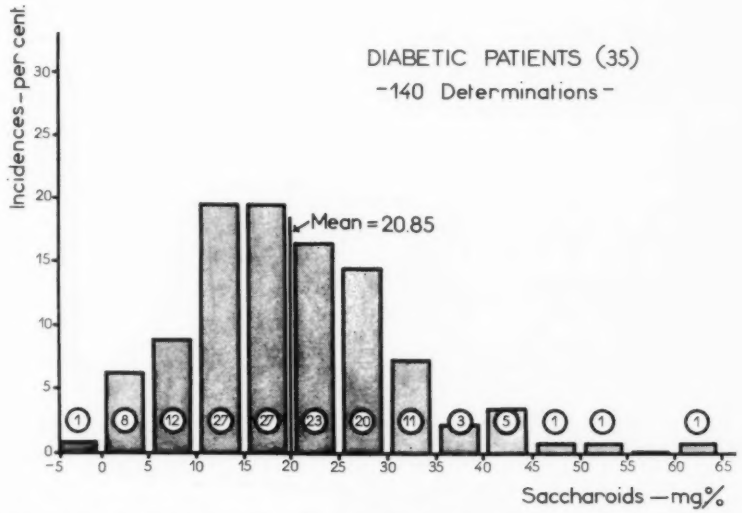

Fig. 2.—Distribution of saccharoid values obtained for a group of thirty-five diabetic patients (140 samples).

Identification of Blood Saccharoid Substances.—The separation and identification of nonglucose reducing substances present in protein-free Somogyi zinc filtrates of blood plasma were attempted as follows: Large venous blood samples (35 to 50 ml.) were drawn from patients with very high as well as from those with normal saccharoid levels. A 1:10 Somogyi zinc sulfate-barium hydroxide filtrate[7] was prepared for each sample, the blood sugar values determined with both the Nelson-Somogyi and glucose oxidase methods, and their saccharoid content calculated. A sample with a saccharoid content of about 30 mg. per cent was chosen as typical of the diabetic bloods, one with a saccharoid content of about 10 mg. per cent as typical of the nondiabetic group. The water from these samples was removed by freeze drying and lyophilization until only a crystalline powder remained. The powders were then dissolved in 1.0 ml. of 10 per cent aqueous isopropanol. Both the normal and diabetic blood filtrates were spotted with a microburet on a large sheet of S & S No. 589 filter paper; 10, 20, and 40 microliters were used for each sample. A 0.5 per cent solution of lactose, galactose, and glucose was spotted on the sheet as a standard. This chromatogram was run for 16 hours in the solvent system, ethyl acetate-pyridine-H_2O (1:2.5:3.5), containing 0.15N $AgNO_3$ in the solvent layer. The paper was air-dried for 1 hour to remove the solvent, exposed to NH_4OH vapors in a sealed chamber for 1 hour, and heated in an oven for 20 minutes at 80° C.

Both samples showed black deposits characteristic of glucose and, as expected, that of the diabetic blood was present in greater quantities. Both samples also contained small amounts of an unknown reducing substance which migrated with an Rf value somewhat

TABLE I. REPRODUCIBILITY OF SACCHAROID VALUES OBTAINED FOR A GROUP OF SIX DIABETIC PATIENTS AT VARIOUS TIME INTERVALS USING CAPILLARY AND VENOUS BLOOD SAMPLES

DATE	1ST SERIES 8/26/58				2ND SERIES 8/29/58				3RD SERIES 9/4/58				4TH SERIES 9/7/58				5TH SERIES 9/10/58			
	CAPILLARY		VENOUS		CAPILLARY		VENOUS		CAPILLARY		VENOUS		CAPILLARY		VENOUS		CAPILLARY		VENOUS	
	N.S.*	G.O.†	N.S.	G.O.	N.S.	G.O.	N.S.	G.O.	N.S.	G.O.	N.S.	G.O.	N.S.	G.O.	N.S.	G.O.	N.S.	G.O.	N.S.	G.O.
Group.—																				
Glucose																				
Mean (mg. %)	206	188	201	183	199	171	197	170	137	122	134	121	149	131	146	128	177	148	175	147
Saccharoids																				
Mean (mg. %)	19		18		28		27		15		13		18		18		29		28	
Patient No. 1.—																				
Glucose																				
Mean (mg. %)	126	121	123	119	92	75	88	73	96	94	95	93	118	108	117	108	147	134	145	131
Saccharoids																				
Mean (mg. %)	5		4		17		15		2		2		10		9		13		14	
Patient No. 6.—																				
Glucose																				
Mean (mg. %)	243	217	234	212	283	252	280	250	186	172	184	171	164	141	164	140	234	188	232	185
Saccharoids																				
Mean (mg. %)	26		22		31		30		14		13		23		24		46		47	

*Nelson-Somogyi.
†Glucose-oxidase.

greater than that of lactose. In an attempt to identify this material, a similar chromatogram was run but *without* $AgNO_3$ solution. The dried chromatogram was dipped in benzidine reagent[8] and then heated in an oven at 105° C. for 10 minutes. With this method spots were obtained for glucose, but the additional unknown was not detected, possibly because of its low concentration. No spots were seen on either chromatogram to indicate the presence of appreciable amounts of aldoses.

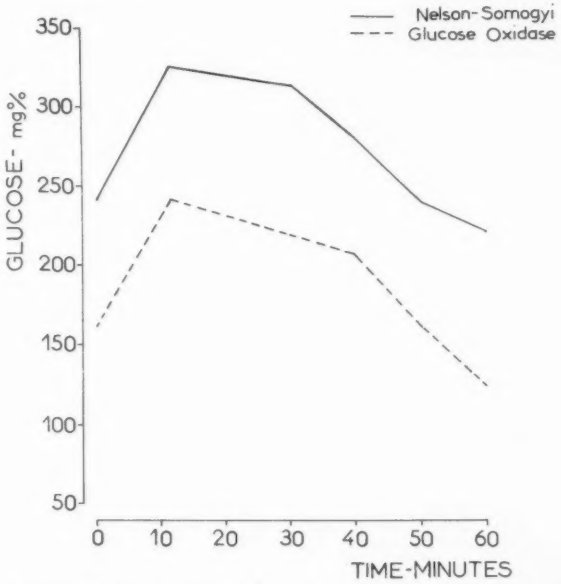

Fig. 3.—Blood glucose values obtained during a modified glucose insulin tolerance test for a diabetic patient with high saccharoid levels with both the Nelson-Somogyi and glucose oxidase procedures.

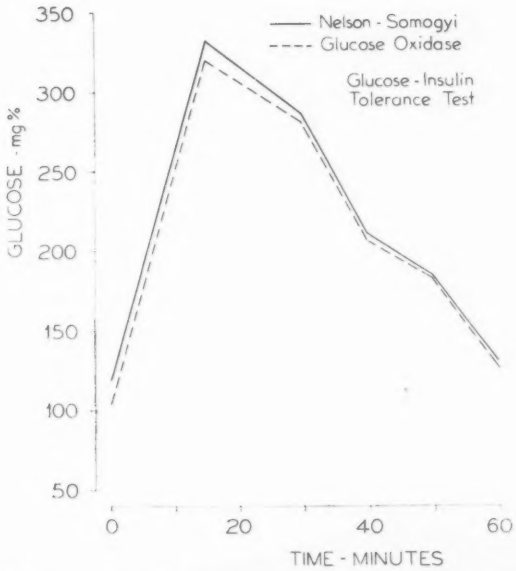

Fig. 4.—Blood glucose values obtained during a modified glucose insulin tolerance test for a diabetic patient with low saccharoid values with both the Nelson-Somogyi and glucose oxidase procedures.

An additional chromatogram was prepared with the use of a sheet of Whatman No. 1 filter paper and spotted with 40 μl. aliquots of normal and diabetic blood filtrates as well as 1 per cent solutions of gluconic, glucuronic, and pyruvic acids as known standards. The chromatogram was developed in the solvent system *N*-butyl alcohol–formic acid–H_2O (2:10:15) for 20 hours. The paper was then air-dried for 3 hours and oven-dried for 30 minutes at 80° C. The chromatogram was developed with 0.2 per cent bromphenol blue in ethanol (pH 5.5) which shows organic acids as yellow spots against a blue background.

In each sample this chromatogram revealed an acid near the origin, having approximately the same Rf value as gluconic and glucuronic acids. The concentration of the acid appeared to be somewhat greater in the diabetic sample than in the normal one. Neither sample contained pyruvic acid.

Effect of Glucose-Insulin Tolerance Test.—Modified glucose-insulin tolerance tests (GITT) were performed on a group of twelve diabetic patients with the technique previously described.[5] The rate of decline for the diabetic patients varied between 1.5 and 3.8 mg. per cent per minute and 4 of the values were 2.5 mg. per cent per minute or less, indicating that these individuals were insulin insensitive. The saccharoid values in the same group varied between 10 and 75 mg. per cent and did not correlate with the calculated rate of decline of the blood sugar. A typical glucose-insulin tolerance curve obtained with capillary blood through the use of both methods is shown in Fig. 3. The curves obtained with each method are parallel, indicating that the saccharoid value is independent of the blood sugar level and is not influenced by either glucose or insulin administration. Results obtained with another diabetic patient with low saccharoid values are illustrated in Fig. 4.

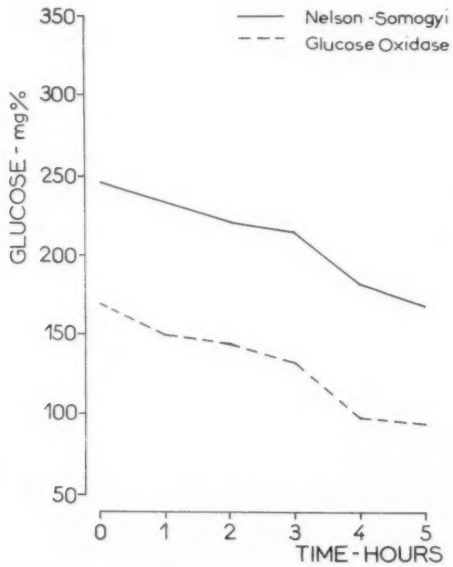

Fig. 5.—Blood glucose values obtained for a 5 hour period (at hourly intervals) for a diabetic patient subsequent to the oral administration of a single 1 Gm. test dose of tolbutamide. The whole blood filtrates were analyzed for glucose with both the Nelson-Somogyi and glucose oxidase methods.

Effect of Tolbutamide Administration.—The same two groups of patients on whom the GITT had been performed also received a single test dose of one Gm. of tolbutamide orally after an 18 hour fast. Capillary bloods were taken just prior to tolbutamide administration and at hourly intervals thereafter for a period of 5 hours. The blood sugars were determined by both procedures for each sample drawn. The curves obtained with each of the two methods were parallel, indicating that the drug has no detectable effect on the

blood saccharoid levels regardless of the severity of the diabetes or of the extent to which the blood sugar is lowered. The results obtained in a typical diabetic patient with elevated saccharoid values are shown in Fig. 5.

DISCUSSION

Benedict[1] reported saccharoid values of 5 to 9 mg. per cent in Somogyi zinc filtrates. Somogyi[9] found an average value of 5 mg. per cent and Folin and Svedberg[10] reported saccharoid levels of 5 to 6 mg. per cent in normal blood. These investigators all used yeast fermentation to remove glucose and copper-reducing methods to measure the saccharoid content in the deproteinized filtrate of the residue. In the present study a mean saccharoid value of 6.0 mg. per cent ± 4.0 S.D. for the nondiabetic group was found. This is in excellent agreement with the values found by the earlier investigators. However, our results do not agree with those of Middleton[11] who found a mean saccharoid value of 27 mg. per cent for normal and of 30 mg. per cent for diabetic patients with the use of venous blood, or a statistically nonsignificant difference between the two groups. For capillary blood he obtained saccharoid values of 37 and of 51 mg. per cent for the normal and diabetic groups, respectively, a highly significant group difference. As previously stated, in the present study no appreciable difference was found between the glucose values of capillary and venous diabetic blood. The mean saccharoid value of 21.0 mg. per cent ± 10.6 S.D. for the diabetic group represents a highly significant difference from the normal values. It should be noted, however, that about 35 per cent of the diabetic patients have saccharoid values that fall into the normal range (0 to 15 mg. per cent). It appears to us that the abnormally high saccharoid values were obtained by Middleton[11] because: (a) iron salts were used for preparation of the whole blood filtrates which contain increased amounts of saccharoids,[12] and (b) the glucose oxidase enzymatic reaction was carried out at pH 5.0 instead of pH 7.0, the latter being the optimal pH for the enzyme systems employed.[3]

In filtrates the substances which may be responsible for copper reduction can be divided into a major component which consists of yeast fermentable D-glucose, and a minor one which is composed of nonglucose reducing substances, i.e., saccharoids. Among the substances mentioned in the literature, those which may contribute to the saccharoid values are: amino acids, homogentisic acid, creatine, uric acid, ergothionine, phenols, ascorbic acid, pentoses, and other nonfermentable carbohydrates, as well as glucuronic acid and some of its metabolites. Many of these compounds, such as ergothionine, are present in red cells to a considerably greater extent than in plasma, and in tungstic acid to a greater extent than in zinc filtrates. Others, such as uric acid and glutathione, are precipitated by some heavy metal cations during the preparation of the protein-free filtrate. Recently developed techniques for the chromatographic separation and identification of carbohydrates have confirmed the fact that ordinarily D-glucose is the only reducing sugar present in blood filtrates[13] in significant amounts. Our studies have confirmed this finding for diabetic as well as for normal blood, but we did find trace amounts

of another, unknown reducing substance. The data of Athanail and Cabaud[14] indicate that at least part of the saccharoids must be a nonfermentable carbohydrate-like substance, since both tungstic acid and zinc filtrates yield the same values with their 2-amino-biphenyl carbohydrate reagent in highly acidic medium. Such a finding would be in agreement with the conclusions of Fashena and Stiff[15] that the "non-glucose, copper reducing fraction of blood, as determined by the Folin-Wu method,[16] can be almost completely accounted for by glutathione and glucuronic acid." The chromatographic studies reported in this paper have confirmed the presence of an organic acid with an Rf value corresponding to glucuronic acid in plasma barium-zinc filtrates. It is known that normal human blood contains 3 to 9 mg. per cent of glucuronic acid in a free or ester conjugated form[17] and that the copper reducing equivalent for glucuronic acid is practically identical with that of glucose.[18] Since glucuronic acid, unlike glutathione, is not precipitated by heavy metal salts, and is nonfermentable by yeast, its presence in zinc filtrates could account for most, if not all, of the saccharoids present in normal plasma.

As previously mentioned, there appears to be chromatographic evidence for an increased amount of an organic acid in the blood barium-zinc filtrates of the diabetic patients with increased saccharoid levels. Whether this increased amount of acid is sufficient to account for differences in copper reduction glucose equivalents of 15 mg. per cent, or greater, in some of the diabetic persons studied, must await a more detailed quantitative investigation of the content of blood glucuronic acid and its metabolites in the diabetic state. At the present time, the possibility cannot be excluded that other reducing substances may also be increased in the diabetic state or that the increased saccharoid level may be due to the presence of an inhibitory substance in the blood of some diabetic individuals.

The GITT demonstrated that acute administration of either glucose or insulin did not influence the level of the blood saccharoids and, furthermore, that this level is independent of the patient's sensitivity to insulin. In addition, it was shown that the saccharoid values are not influenced by tolbutamide, despite the marked reduction in "true" glucose level during the 5 hour interval after its oral administration. This finding is in accord with the view that this drug acts by means of stimulation of pancreatic insulin output.[19-22] Other workers have also demonstrated that the saccharoid values are independent of the blood sugar level.[10, 11, 23] Smelo and co-workers[23] concluded from their studies that the saccharoid content of normal individuals is independent of change in diet and of diurnal glycemic fluctuations and is relatively consistent over long periods of time, but that the level can be reduced by fasting. The results of the present study are in general agreement with these findings, although the diabetic patients showed much wider fluctuations than the nondiabetic individuals (Table I).

It has been suggested by Marks[24] that control of the patient's carbohydrate metabolism by means of insulin or other therapy (fasting, tolbutamide, etc.) over prolonged periods of time may cause an eventual return of an elevated saccharoid level to normal values. If this is borne out, the deter-

mination of the saccharoid level of a diabetic patient would then provide a good laboratory index of the status of the carbohydrate metabolism in this disease. Furthermore, the elevation of the level of glucuronic acid or its metabolites in the diabetic person would seem to indicate that in the presence of a deranged carbohydrate metabolism, there is increased carbohydrate utilization via the hexose monophosphate shunt, in which glucuronic acid is one of the intermediaries.[25]

SUMMARY

1. The blood saccharoid levels of twenty nondiabetic subjects (80 samples) and of thirty-five diabetic patients (140 samples) were determined as the difference between the Nelson-Somogyi copper-reduction method and the glucose oxidase method. No significant difference was found between the saccharoid values of capillary and venous blood for either the diabetic or nondiabetic groups.

2. The mean normal saccharoid level was 6.0 mg. per cent ± 4.0 S.D. and for the diabetic group the mean value obtained was 21.0 mg. per cent ± 10.6 S.D.

3. The saccharoid concentrations of twelve diabetic and six nondiabetic individuals were determined during the modified glucose-insulin tolerance test and during the 4 hour interval after a single oral 1 Gm. dose of tolbutamide. No changes were observed in the saccharoid levels in the course of these tests. The elevation of the saccharoid level of the diabetic was not related to insulin sensitivity, as determined by this test.

4. An attempt was made by paper chromatographic techniques to determine the saccharoid substances present in barium-zinc filtrates which are responsible for the increased copper reduction values. An organic acid was found in increased amounts in such filtrates which had the same Rf value as glucuronic acid.

5. The possible importance in some diabetic patients of alternate pathways of carbohydrate metabolism which might result in an increased production of blood glucuronic acid or other similar reducing metabolites (saccharoids) is suggested. Furthermore, the use of saccharoid levels as an index of metabolic control of diabetes is considered.

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REFERENCES

1. Benedict, S. R.: Analysis of Whole Blood. II. Determination of Sugar and of Saccharoids (Non-fermentable Copper-Reducing Substances), J. Biol. Chem. **92**: 141, 1931.
2. Nelson, N.: Photometric Adaptation of the Somogyi Method for the Determination of Glucose, J. Biol. Chem. **153**: 375, 1944.
3. Saifer, A., and Gerstenfeld, S.: Photometric Microdetermination of Blood Glucose With Glucose Oxidase, J. LAB. & CLIN. MED. **51**: 448, 1958.
4. Cawley, L. P., Spear, F. E., and Kendall, R.: Ultramicro Chemical Analysis of Blood Glucose With Glucose Oxidase, Am. J. Clin. Path. **32**: 195, 1959.

5. Lazarus, S. S., and Volk, B. W.: Estimation of Insulin Sensitivity by the Modified Glucose Insulin Tolerance Test, J. LAB. & CLIN. MED. **39**: 404, 1952.
6. Volk, B. W., and Lazarus, S. S.: Effect of Varying Insulin Dosages on the Rate of Decline of the Blood Sugar in the Modified Glucose Insulin Tolerance Test, J. LAB. & CLIN. MED. **41**: 684, 1953.
7. Somogyi, M.: Determination of Blood Sugar, J. Biol. Chem. **160**: 69, 1945.
8. Harris, G., and MacWilliams, I. C.: Dipping Technique for Revealing Sugars on Paper Chromatograms, Chem. & Indust., p. 249, 1954.
9. Somogyi, M.: Distribution of Blood Sugar, J. Biol. Chem. **90**: 731, 1931.
10. Folin, O., and Svedberg, A.: Sugar in Urine and in Blood, J. Biol. Chem. **70**: 405, 1926.
11. Middleton, J. E.: Experience With a Glucose-Oxidase Method for Estimating Glucose in Blood and C.S.F., Brit. M. J. **1**: 824, 1959.
12. MacLean, H.: Glucolysis in Diabetic Blood With a Method for the Estimation of Blood Sugar, J. Physiol. **50**: 168, 1916.
13. Bell, D. J.: Tissue Components of the Domestic Fowl. I. The D-Glucose Content of Whole Blood, Biochem. J. **66**: 137, 1957.
14. Athanail, G., and Cabaud, P. G.: Simplified Colorimetric Method for True Blood Glucose, J. LAB. & CLIN. MED. **51**: 321, 1958.
15. Fashena, G. J., and Stiff, H. A.: Nature of Saccharoid Fraction of Human Blood. II. Identification of Glucuronic Acid, J. Biol. Chem. **137**: 21, 1941.
16. Folin, O., and Wu, H.: System of Blood Analysis; Simplified and Improved Method for Determination of Sugar, J. Biol. Chem. **41**: 367, 1920.
17. Fishman, W. H., Smith, M., Thompson, D. B., Bonner, C. D., Kasdon, S. C., and Homburger, F.: Investigation of Glucuronic Acid Metabolism in Human Subjects, J. Clin. Invest. **30**: 685, 1951.
18. Hawk, P. B., Oser, B. L., and Summerson, W. H.: Practical Physiological Chemistry, ed. 13, Philadelphia, 1954, Blakiston Company, p. 842.
19. Loubatières, A.: Analyse du méchanisme de l'action hypoglycémiante du *P*-amino-benezenesulfamidothiodiazol (2254 RP), Compt. rend. Soc. biol. **138**: 766, 1944.
20. Colwell, A. R., Jr., Colwell, J. A., and Colwell, A. R., Sr.: Intrapancreatic Perfusion of the Antidiabetic Sulfonylureas, Metabolism **5**: 749, 1956.
21. Lazarus, S. S., and Volk, B. W.: Comparison in Rabbits of Hypoglycemia and B Cell Degranulation After Various Sulfonylureas, Proc. Soc. Exper. Biol. & Med. **102**: 67, 1959.
22. Volk, B. W., and Lazarus, S. S.: Pathogenesis of Orinase-Induced Beta-Cell Degranulation, Diabetes **7**: 125, 1958.
23. Smelo, L., Kern, F. M., and Drabkin, D. L.: Determination of Saccharoids and the Relationship of the Saccharoid Content of the Blood to Diet and Drugs, J. Biol. Chem. **125**: 461, 1938.
24. Marks, V.: An Improved Glucose-Oxidase Method for Determining Blood, C.S.F. and Urine Glucose Levels, Clin. chim. acta **4**: 395, 1959.
25. Eisenberg, F., Jr., Dayton, P. G., and Burns, J. J.: Studies on the Glucuronic Acid Pathway of Glucose Metabolism, J. Biol. Chem. **234**: 250, 1959.

SIGNIFICANCE OF VASCULAR INJURIOUS FACTORS IN HUMAN PLASMA AS DETERMINED BY ANIMAL ASSAY

II. Role of Human Complement in Increasing Capillary Permeability

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In the past, bioassay for vascular injurious factors in human plasma have generally been performed in laboratory animals. Since normal human plasma evokes capillary vascular injury in heterologous species, bioassay of human plasma holds little meaning until the nonspecific toxicity for heterologous species can be characterized and eliminated. This report is concerned with factors in normal human plasma which increase heterologous capillary permeability. It was found that inactivation of human complement by heat, storage, or ether extraction significantly reduced the peritoneal capillary permeability promoting activity of fresh human plasma for the rat. Comparable results were obtained when human complement was inactivated with immune precipitates, zymosan, ammonia, streptokinase, or by dialysis against distilled water. The precise mechanism of complement action could not be defined: absorption of human plasma with washed rat erythrocytes, platelets, or macerated abdominal tissues failed to impair the capillary permeability promoting activity; Forssman antibody or human properdin could not be implicated; peritoneal mast cell injury did not appear responsible. Inactivation of human complement did not completely abolish the peritoneal capillary permeability promoting activity of human plasma. The residual capillary permeability promoting activity factors were not dialyzable and were concentrated in the globulin fraction. While the primary mechanisms responsible for the capillary permeability promoting activity of fresh normal human plasma in the rat peritoneal cavity do not appear related to those described by Miles and associates, or to those which increase cutaneous capillary permeability in the nonsensitized rabbit, they do resemble those reported to injure cutaneous capillary vessels in the nonsensitized guinea pig.

Humoral factors capable of damaging the capillary vascular system have frequently been sought during various human illnesses. In most such studies, assays for vascular injurious factors in human plasma or serum have been conducted in laboratory animals. Fresh *normal* human plasma, however, evokes capillary injurious effects in nonsensitized heterologous species such as the rabbit and the rat. This is made evident by the appearance of prolonged capillary dilatation, decreased vasomotor activity, depression of terminal arteriolar reactivity to topically applied epinephrine, and increased capillary permeability.[1] Such action of human plasma appears related primarily to the heterologous nature of the assay systems and can be intensified significantly

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either by 48 hour starvation of the human donor or by allowing the donor to ingest large meals which are rich in fat.[1] The nonspecific capillary injurious effects of normal human plasma for the experimental animal and the enhancement of these noxious effects by alterations in human nutritional status increases the complexity of human plasma assay and necessitates a re-evaluation of the significance of those humoral vascular injurious factors previously reported as characteristic for certain human illnesses.[2-5]

The present studies were initiated to consider the mechanisms whereby normal human plasma damages the capillary vascular system of the nonsensitized experimental animal. The elucidation of such mechanisms may permit the elimination of the nonspecific vascular injurious effects of human plasma; characterization of those humoral factors specific to various disease entities might then be accomplished. The following report deals with the mechanisms whereby human plasma increases capillary vascular permeability when injected into nonsensitized heterologous species. The findings indicate that in the heterologous host human complement may play an important role in mediating an increase in capillary permeability.

MATERIALS AND METHODS

Collection of Human Plasma.—Blood (25 to 50 ml.) from normal subjects was drawn into sterile syringes containing sufficient heparin to provide 25 μg per milliliter blood. The blood was transferred to iced sterile tubes, immediately spun at 2,000 RPM, for 15 minutes at 4° C., and the plasma held at 4° C. or −10° C. until assay.

Assay for Capillary Permeability Enhancement.—The method for determination of increases in capillary permeability induced by human plasma was modified slightly from that described by Lake and co-workers.[6] Normal fasting albino, male rats, never previously exposed to human or other foreign sera, and weighing 80 to 150 grams, were utilized throughout. The rats were lightly anesthetized with ether before a small skin tag was cut from the midline anterior abdominal wall. The peritoneal lining was gently elevated with blunt forceps, with especial care taken to avoid including segments of intestine. Human plasma, or the appropriate control, was then injected through the elevated peritoneum via a 25 gauge needle; the fluid volume injected was kept constant at 20 ml. per kilogram of rat weight. The entire procedure required less than one minute, and the anesthesia was immediately discontinued. The rats were permitted to move freely thereafter for 3 hours and then sacrificed by decapitation. The abdominal cavity was opened rapidly, the peritoneal fluid carefully removed with a Pasteur pipette, transferred to measuring pipettes, and the volume recorded. For reasons already published (and reviewed in the present discussion), this volume was employed as an index of the ability of human plasma to increase peritoneal capillary permeability.[6] All plasma assays were performed simultaneously in groups of 3 rats, and the mean values utilized as the representative figures.

Complement Assay.—Aliquots of a 1:20 dilution of human plasma ranging from 0.10 to 0.50 ml. were placed in 12 by 75 mm. serologic tubes and sufficient phosphate buffer, pH 7.4, added to bring the volume to 2.0 ml.; 0.5 ml. of a 1:4,000 sheep cell amboceptor and 0.5 ml. of washed sheep erythrocytes (2 per cent final concentration) were added to each tube. The tubes were shaken and incubated at 37° C. for 30 minutes. The 50 per cent hemolytic units were calculated as described by Kabat and Mayer.[7]

Absorption of Heterophile Antibodies.—(1) Aliquots of human plasma were incubated for 15 minutes at 37° C. with equal volumes of packed washed rat erythrocytes from the same animals utilized for assay. Absorption was repeated to remove all detectable rat erythrocyte agglutinins. (2) Aliquots of fresh human plasma (2.5 ml.) were absorbed at

37° C. for 45 minutes with washed rat platelets obtained from 10 ml. samples of fresh heparinized rat blood processed in iced siliconed syringes and tubes and concentrated by differential centrifugation at 4° C. as described by Quick and Epstein.[8] (3) Samples of human plasma were incubated 15 minutes at 37° C. with equal volumes of *washed* freshly macerated rat abdominal viscera, including most of the peritoneal lining. The tissues were macerated either with a mortar and pestle or by hemogenation in a Waring Blendor. In each of the above studies, control human plasma aliquots were incubated and assayed simultaneously.

Inactivation of Human Complement.—The methods other than heating, storage, or extraction with ether employed for inactivation of human complement are indicated below; hemolytic complement titrations were performed prior to and after each procedure to ascertain the extent of inactivation: (1) Immune precipitates obtained from the interaction of horse serum and antihorse rabbit serum were washed 3 times and sufficient quantities incubated overnight at 4° C. with aliquots of fresh human plasma so as to reduce markedly the hemolytic complement titer. (2) One hundred units streptokinase (Varidase) were added to each milliliter of human plasma and incubated at 37° C. for 1 hour. (3) Fresh human plasma specimens were incubated with zymosan (Fleischman), 2 mg. per milliliter for one hour at 37° C. The tubes were agitated frequently and the zymosan removed by centrifugation at 4,000 RPM for 45 minutes at 4° C. (In each of the above studies, control samples of human plasma were handled comparably and assayed simultaneously.) (4) Human plasma aliquots were treated with ammonia as described by Kabat and Mayer.[7] Controls were run with isotonic saline substituted for ammonium hydroxide. (5) Aliquots of human plasma were dialyzed against distilled water overnight at 4° C., with constant magnetic stirring. Controls were similarly dialyzed against isotonic saline solutions.

RESULTS

The intraperitoneal injection of 20 ml. per kilogram of isotonic saline, distilled water, or normal rat plasma into normal albino rats failed to induce any symptoms of systemic illness or of peritoneal irritation. The volume of fluid obtained from the peritoneal cavity 3 hours later did not exceed 2.3 ml. per kilogram (Table I), and vascular congestion of the peritoneum was never ob-

TABLE I. VOLUME OF RAT PERITONEAL FLUID RECOVERED (ML./KG.) 3 HOURS AFTER INTRAPERITONEAL INJECTION OF VARIOUS FLUIDS (20 ML./KG.)

Isotonic saline	1.5	1.8	2.1	
Distilled water	1.7	1.5		
Normal rat plasma	1.7	2.0	2.3	1.4

Each result represents the mean obtained in groups of three rats.

served in the gross. In contrast, the intraperitoneal injection of fresh human plasma from thirty-nine normal subjects into nonsensitized albino rats consistently evoked characteristic symptoms. Within several minutes the normal activity slowed, the fur became ruffled, and the hind limbs were stretched repeatedly; respiratory rate increased, the act of walking appeared painful, and after 5 to 20 minutes the animal lay quietly or fell with the legs bent upward on the abdomen. The severity of these symptoms varied with the individual plasma but were reproducible with the same plasma. Upon sacrifice 3 hours later, the rat peritoneal lining was grossly congested. The volume of peritoneal fluid at this time varied, depending upon the human plasma specimen injected. Whereas 20 ml. per kilogram rat weight of human plasma was injected intraperitoneally, from 14.2 to 54.2 ml. per kilogram of fluid was now present. With

any given human plasma specimen, the amount of fluid in groups of rats was reproducible, generally falling within ± 10 per cent of the mean.

Effect of Human Complement Inactivation.—The effect of the established and consistent methods of inactivating complement, i.e., heating human plasma at 56° C. for 30 minutes, storage, and ether extraction, are indicated in Table II.

TABLE II. EFFECT OF HEAT, STORAGE, AND ETHER EXTRACTION OF HUMAN PLASMA ON VOLUME OF RAT PERITONEAL FLUID 3 HOURS AFTER INTRAPERITONEAL INJECTION (20 ML./KG.)

HUMAN PLASMA SPECIMEN NO.	PERITONEAL FLUID RECOVERED* (ML./KG.)			
	UNTREATED	HEATED 56° C. FOR 30 MIN.	STORAGE AT 4° C.	ETHER EXTRACTED AT 0° C.
86	48.5	17.7		
87	35.3	10.4		
88	19.1	2.5		
89	35.2		27.4 (12 days) 17.7 (30 days)	
90	39.6			10.0
91	34.0			13.4
92	31.2			9.7
93	25.8			7.6

*Each result represents the mean obtained in groups of three rats.

TABLE III. EFFECT OF INACTIVATION OF HUMAN AND RABBIT PLASMA COMPLEMENT ON RAT PERITONEAL FLUID 3 HOURS AFTER INTRAPERITONEAL INJECTION (20 ML./KG.)

HUMAN PLASMA SPECIMEN NO.	PERITONEAL FLUID RECOVERED* (ML./KG.)							
		METHODS OF COMPLEMENT INACTIVATION						
	CONTROL	IMMUNE PRECIPITATE	ZYMOSAN		NH_3	STREPTOKINASE	DIALYSIS AGAINST DISTILLED WATER
			18° C.	37° C.			
103	30.0 (82)†	7.4 (17)					
104	34.9 (86)	4.6 (12)					
105	40.0 (89)		30.0 (68)	8.0 (25)			
106	33.4 (66)		31.5 (58)	8.6 (8)			
107	44.0 (78)		50.0 (75)	8.7 (19)			
108 (adjusted to ionic strength 0.4)	48.0 (79)			45.0 (79)			
108A (above heated 56° C. for 30 min.)	5.7 (0)						
109	23.0 (54)				7.5 (0)		
110	54.0 (90)					22 (30)	
111	31.6					8.2	
112 (rabbit)	11.0 (15)					11.0 (15)	
113 (rabbit)	17.4					15.4	
114	31.0 (73) (dialized against isotonic saline)						3.0 (0)

*Each result represents the mean obtained in groups of three rats.

†Figures in parentheses represent human complement titers expressed as 50 per cent sensitized sheep cell hemolytic units.

The peritoneal fluid recovery was always reduced significantly, although not abolished, by each of these treatments. The effects of inactivating human complement by more specific methods are indicated in Table III. The reduction of human plasma hemolytic complement titer with immune precipitates, streptokinase, zymosan, ammonia, or dialysis against distilled water led to marked reductions, again without abolition, of the peritoneal fluid volume recovery. To evaluate the specificity of action of zymosan, the ionic strength of human plasma was increased initially to 0.4 with sodium chloride to prevent complement inactivation[9]; this manipulation inhibited both the complement inactivation and the fall in peritoneal fluid volume (Table III, Specimen 108). To ascertain that the high ionic strength of the plasma per se was not responsible for the increased peritoneal volume, the human plasma, adjusted to ionic strength 0.4, was heat inactivated; the peritoneal volume then decreased sharply (Table III, Specimen 108A). To evaluate the specificity of action of streptokinase, the effect was tested on rabbit plasma. This addition failed to reduce the hemolytic complement titer or the peritoneal fluid volume recovered (Table III, Specimens 112 and 113). In four trials, mixing heat inactivated human plasma with equal volumes of fresh heparinized rat plasma failed to enhance the peritoneal fluid recovery.

TABLE IV. EFFECT OF ABSORPTION OF HUMAN PLASMA WITH WASHED RAT ERYTHROCYTES, PLATELETS, AND MACERATED ABDOMINAL VISCERA ON VOLUME OF RAT PERITONEAL FLUID 3 HOURS AFTER INTRAPERITONEAL INJECTION (20 ML./KG.)

HUMAN PLASMA SPECIMEN NO.	PERITONEAL FLUID RECOVERED* (ML./KG.)			
		HUMAN PLASMA ABSORBED WITH WASHED RAT		
	CONTROL	ERYTHROCYTES	PLATELETS	VISCERA
94	15.8 (80)†	20.0 (75)		
95	14.2 (76)	15.6 (72)		
96	36.2 (68)	28.3 (60)		
97	18.0	20.6		
98	41.0 (75)		40.0 (60)	
99	17.7 (78)			17.7 (75)
100	27.0			25.0

*Each result represents the mean obtained in groups of three rats.

†Figures in parentheses represent human complement titers expressed as 50 per cent sensitized sheep cell hemolytic units.

Effect of Heterophile Antibody Absorption.—Absorption of human plasma with washed rat erythrocytes, platelets, or macerated abdominal viscera failed to reduce significantly the hemolytic complement titers or the peritoneal fluid volume recovery (Table IV). Absorption of human plasma with rat erythrocytes resulted in no hemolysis; thus it appeared unnecessary to check the influence of hemolysis on peritoneal fluid volume. It also seemed unlikely that serotonin released during platelet absorption might be responsible for maintenance of the peritoneal fluid volume, since addition of 6.0 μg serotonin (freshly prepared and physiologically active in vascular perfusion experiments) to each milliliter of three control aliquots of the human plasma enhanced mean rat peritoneal fluid volume but slightly, from 41.0 to 45.2 ml. per kilogram.

Effect of Anticoagulant in Human Plasma.—Although heparin possesses anticomplementary activity, this effect operates when large amounts, out of all

proportion to its anticoagulative properties, are employed.[10] With the dosage of heparin employed in the present experiments, approximately 50 μg per milliliter of plasma, the inhibition of hemolytic complement is slight, falling between 2 and 5 per cent.[10] That the heparin did not affect the volume of peritoneal fluid obtained was confirmed by finding that fresh human serum, as well as citrated plasma (1 ml. of 3.8 per cent sodium citrate per 9 ml. blood) possessed activity comparable to that of the fresh corresponding heparinized plasma specimens.

Effect of Dilution.—Dilution of human plasma with isotonic saline induced a progressive and arithmetic reduction in the peritoneal fluid volume recovered (Fig. 1). Furthermore, similar dilution of homologous rat plasma failed to unmask any factor which increased peritoneal volume (Fig. 1).

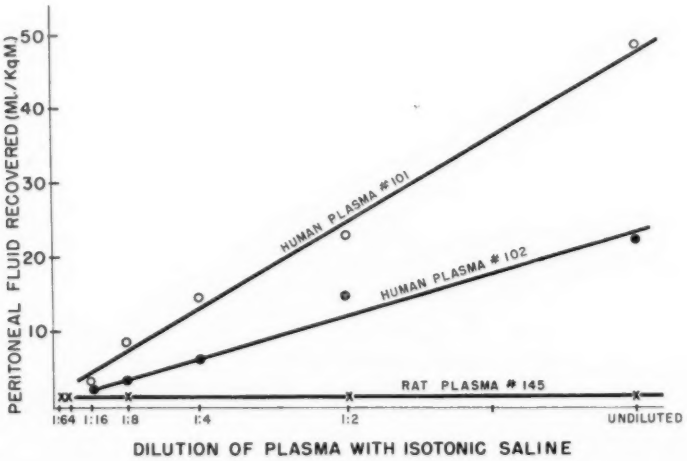

Fig. 1.—The effect of dilution of human and rat plasma with 0.85 per cent saline on the fluid volume recovered from the rat peritoneal cavity three hours after intraperitoneal innoculation of 20 ml./Kg. (Each result represents the mean obtained in groups of three rats.)

Effect of Properdin Removal.—Reduction in human serum properdin levels, achieved by incubating serum with zymosan at 18° C., did not impair the peritoneal volume recovered (Table III, Specimens 105 through 107). Only those specimens exhibiting less than a 25 per cent reduction in original hemolytic complement titers following properdin depletion were considered. That depletion of properdin had been accomplished was indicated by the minimal decreases in complement levels upon reincubation of the serum with fresh zymosan at 37° C., in contrast to the nondepleted serum aliquots. The precise reductions in serum properdin titers were not quantitated.

Effect of Human Albumin and Globulin Fractions.—Freshly drawn human plasma was fractionated by dialysis against ammonium sulfate solutions overnight at 4° C., with magnetic stirring. The precipitated fractions were then washed, suspended in phosphate buffered isotonic saline (pH 7.4), and dialyzed against the same buffer until free of ammonium ion, and the volumes reconstituted with buffer to equal that of the initial plasma sample. The fraction

obtained at 50 per cent ammonium sulfate saturation led to greater peritoneal volume recoveries than that obtained between 50 and 100 per cent saturation. However, neither fraction, nor their recombination, exhibited the activity of the whole plasma (Table V). None of the fractions or the recombined aliquots possessed detectable hemolytic complement.

TABLE V. VOLUME OF RAT PERITONEAL FLUID 3 HOURS AFTER INTRAPERITONEAL INJECTION OF HUMAN PLASMA ALBUMIN AND GLOBULIN FRACTIONS (20 ML./KG.)*

	PERITONEAL FLUID RECOVERED† (ML./KG.)				
HUMAN PLASMA SPECIMEN NO.	UNTREATED CONTROL	HEAT INACTIVATED	50% AMMONIUM SULFATE FRACTION (A)	50 to 100% AMMONIUM SULFATE FRACTION (B)	A + B 1:1
118	47.3	12.2	9.5	5.0	14.0
119	27.8	7.8	10.8	2.8	6.7

*Plasma fractions prepared at 4° C. by dialysis against ammonium sulfate, dialyzed free of ammonium ion at 4° C., and reconstituted in buffered isotonic saline to original plasma volume.

†Each result represents the mean obtained in groups of three rats.

Effect of Serotonin Inhibitors.—Treatment of five groups of rats (three per group) with 7 mgm. per kilogram intravenous dibenamine, 15 minutes prior to injection of fresh human plasma failed to alter significantly the peritoneal fluid volume recovered. Pretreatment of four groups of rats with intravenous Phenergan, 16 mg. per kilogram, also failed to alter the peritoneal volume following the injection of fresh human plasma or the residual volume recovered after injection of heat inactivated plasma.

DISCUSSION

When fresh normal human plasma or serum is injected intraperitoneally into nonsensitized rats, rabbits, or guinea pigs, the volume of peritoneal fluid 3 hours later significantly exceeds that which follows isotonic saline or homologous plasma injections.[11] This peritoneal exudation is not peculiar to human plasma; similar results are obtained with other fresh heterologous mammalian plasma. Studies based upon tagged plasma proteins indicate that such peritoneal fluid accumulation is secondary to increased peritoneal capillary permeability; if capillary permeability were not increased, the plasma would be rapidly and wholly absorbed via the abdominal lymphatics.[12] The nature of the substances in heterologous plasma that increase peritoneal capillary permeability has been studied by Lake and associates.[6] These investigators, utilizing the rat as the test animal, found the peritoneal capillary permeability promoting activity of human and rabbit plasma to be present in serum, not removed by dialysis, and impaired by heating at 61° C. for 7 minutes, by storage (faster at 4° C. than at −23° C.), or by ether extraction. Although Lake and associates suggested that an antibody-antigen reaction might be responsible for the peritoneal capillary permeability promoting activity of heterologous plasma, no supporting evidence was obtained. The authors instead concluded that some serum lipoprotein was probably the responsible permeability enhancing factor.

The lipoprotein hypothesis for heterologous capillary permeability promoting activity of human plasma has recently been questioned. Thus the lipoprotein fraction of human plasma prepared by the Gofman technique of ultracentrifugation was shown to possess minimal peritoneal capillary permeability promoting activity in the rat when compared with the corresponding whole plasma activity.[1] Moreover, since the peritoneal capillary permeability promoting activity of human plasma appears to depend upon the heterologous nature of the assay system, any working hypothesis must consider an immunologic mechanism. The properties of the permeability enhancing factor in human plasma described by Lake and co-workers and confirmed during these studies are compatible not only with a lipoprotein, but more specifically with complement. Evidence was therefore sought to determine whether complement was indeed one of the responsible factors.

The present findings strongly suggest that human complement is an important factor for the heterologous capillary permeability promoting activity of human plasma, at least in the rat peritoneal cavity. Thus inactivation of human hemolytic complement by incubation of human plasma with washed immune precipitates significantly impaired the peritoneal capillary permeability promoting activity. Since these experiments did not exclude the possibility of a nonspecific absorption of capillary permeability promoting activity, corroborative studies were performed. Complement was inactivated by dialysis of human plasma against distilled water, by zymosan treatment at 37° C., by ammonia, and by streptokinase. Regardless of the mode of inactivation, the peritoneal capillary permeability promoting activity was reduced significantly when compared with the corresponding control plasma aliquots possessing the intact hemolytic complement titer. The possibility that zymosan nonspecifically absorbed the capillary permeability promoting activity from human plasma was minimized by testing the effect of increasing plasma ionic strength; at ionic strength of 0.4 neither the hemolytic complement titer nor the plasma capillary permeability promoting activity was reduced. The possibility that streptokinase impaired this activity of human plasma by some mechanism unrelated to complement inactivation was also tested. In contrast to human plasma, small quantities of streptokinase do not inactivate rabbit complement[13]; that streptokinase did not act directly to impair the capillary permeability promoting activity of human plasma was indicated by the failure of streptokinase to reduce this permeability activity of rabbit plasma for the rat.

The manner by which human complement increases peritoneal capillary permeability in the rat could not be defined. A responsible antibody in human plasma was not demonstrable. Thus Forssman antibody could not be implicated because rat tissues do not contain Forssman antigen.[14] Moreover, absorption of heterophile rat erythrocyte or platelet agglutinins from human plasma failed to affect significantly this peritoneal capillary permeability activity or the hemolytic complement titers of the plasma. Serotonin released from the rat platelets during absorption could not account for maintenance of the capillary permeability promoting activity as indicated by the minimal increase in

it which was produced by addition of 6.0 μg serotonin to each milliliter of plasma; this represents approximately twice the serotonin contained in 10 ml. rat blood.[15] Since rat platelets do not contain detectable histamine,[15] platelet histamine release also could not explain the persistence of the capillary permeability promoting activity of absorbed human plasma. However, mast cells in the rat peritoneal cavity contain relatively large quantities of histamine and serotonin[16]; injury to these cells with release of these substances might increase local capillary permeability. Unfortunately, this attractive hypothesis seems untenable since pretreatment of rats with the antiserotonin compound, dibenamine, in dosages reported capable of mitigating increased capillary permeability by mast cell disruption in rat subcutaneous tissues,[17] failed to alter significantly the peritoneal capillary permeability promoting activity of human plasma. Moreover, although pretreatments of rats with antihistaminic as well as antiserotonin agents completely inhibit the local increase in capillary permeability produced by subcutaneous mast cell disruption in the rat,[17] Phenergan, which possesses both antihistaminic and antiserotonin activity,[18] also failed to reduce the capillary permeability promoting activity of human plasma. Attempts to implicate human properdin as a mediator of this activity were similarly unsuccessful.

Whenever complement in human plasma was inactivated, the rat peritoneal capillary permeability promoting activity was markedly reduced but not abolished. Regardless of the method of complement inactivation, significant residual capillary permeability promoting activity was evident, usually approximating 20 to 30 per cent of the original plasma activity. The principle responsible for the residual capillary permeability promoting activity was not dialyzable and was concentrated in the globulin moiety of plasma as fractionated with ammonium sulfate. Although the nature and mechanism of action of the factors in inactivated human plasma are unknown, peritoneal mast cell injury is probably not essential, since Phenergan failed to suppress residual capillary permeability promoting activity.

While human complement appears necessary for maximum capillary permeability promoting activity of human plasma in the rat peritoneal cavity, there was no precise correlation between it and the complement level as measured by the sensitized sheep cell hemolytic method. Indeed, marked variations in the activity existed between plasma specimens drawn from different subjects despite similar hemolytic complement titers. Since some of these variations were too great to be accounted for simply by differences in residual activity after complement inactivation, the findings suggest that human complement may act in direct conjunction with other human plasma factors.

Miles and Wilhelm[19] described several factors in mammalian plasma capable of increasing capillary permeability upon intradermal injection into *homologous* species. One factor, activated by dilution with isotonic saline, has been reported in normal human plasma[20]; a second factor is activated by storage of the plasma. Since dilution or storage of human plasma resulted in a progressive decline in rat peritoneal capillary permeability promoting activity, and since dilution of

homologous rat plasma failed to unmask any such peritoneal activity, it seems unlikely that the factors described by Miles and Wilhelm were responsible for the peritoneal capillary permeability promoting activity of human plasma in the rat.

Several previous studies have considered the ability of normal human plasma or sera to increase heterologous capillary permeability. Thus, fresh undiluted human sera not only increase capillary permeability of nonsensitized guinea pig skin,[21] but also induce histologic evidence of vascular injury.[22] As in the present studies, one factor required for this injurious activity resembles human complement; furthermore, activity could not be correlated neatly with the hemolytic complement titer, nor could it be restored to inactivated aliquots by addition of fresh homologous sera.[22] In contrast, the capillary permeability promoting activity of normal human sera for the nonsensitized rabbit skin has been attributed to its fibrinolytic activity.[23] Activation of this system with streptokinase in the present studies, however, sharply reduced the rat peritoneal capillary permeability promoting activity of human plasma. Moreover, in the rabbit skin, unlike the effect on the rat peritoneum, human complement inactivation by heat or storage does not impair this activity[24]; plasma dilution can be carried as high as 10^6 with isotonic saline before capillary permeability promoting activity is lost,[1] and Phenergan is inhibitory.[6] It would seem that the *primary* mechanisms responsible for the capillary permeability promoting activity of normal human plasma on the rat peritoneum are, at least in part, different from those responsible for the cutaneous capillary permeability promoting activity in the rabbit, but may be similar to those which operate in guinea pig skin.

SUMMARY

Inactivation of human complement by heating, storage, or ether extraction significantly reduced the peritoneal capillary permeability promoting activity of human plasma for the rat. Comparable results were obtained when human complement was inactivated with immune precipitates, zymosan, ammonia, streptokinase, or by dialysis against distilled water. The manner by which human complement increases capillary permeability could not be defined. The mechanism of the capillary permeability promoting activity of human plasma in the rat peritoneal cavity in relation to such activity in other species is considered.

REFERENCES

1. Greisman, S. E., and Wisseman, C. L., Jr.: Significance of Vascular Injurious Factors in Human Plasma as Determined by Animal Assay. I. Influence of Nutritional Status, J. LAB. & CLIN. MED. **56**: 355, 1960.
2. Shorr, E.: Fourth Conference, Factors Regulating Blood Pressure, New York, 1950, Josiah Macy, Jr., Foundation.
3. Shorr, E., Baez, S., Zweifach, B. W., Payne, M. A., and Mazur, A.: The Antidiuretic Action of the Hepatic Vasodepressor Ferritin (VDM) and Its Occurrence in Conditions Associated With Antidiuresis in Man, Trans. A. Am. Physicians **63**: 39, 1950.
4. Shorr, E.: Hypertension: A Symposium, 1951, Univ. Minnesota Press.
5. Shorr, E.: Derangements in VEM-VDM (Ferritin) Metabolism in Hypertension of Cushing's Syndrome; Their Correction During Restoration of Normotension by Bilateral Adrenalectomy, Trans. A. Am. Physicians **65**: 248, 1952.

6. Lake, B. J., Simmonds, W. J., and Steinbeck, A. W.: The Nature of the Capillary Damaging Factor in Foreign Plasma, Australian J. Exper. Biol. & M. Sc. **31**: 65, 1953.
7. Kabat, E. A., and Mayer, M. M.: Experimental Immunochemistry, Springfield, Illinois, 1948, Charles C Thomas, Publisher.
8. Quick, A. J., and Epstein, E.: Thromboplastic Activity in Human Blood, J. Appl. Physiol. **4**: 840, 1952.
9. Pillemer, L., Blum, L., Lepow, I. H., Ross, A. O., Todd, E. W., and Wardlaw, A. C.: The Properdin System and Immunity. I. Demonstration and Isolation of a New Serum Protein, Properdin, and Its Role in Immune Phenomena, Science **120**: 279, 1954.
10. Walton, K. W., Ellis, H. A., and Taylor, C. E. D.: A Method for the Determination of the Anticomplementary Activity of Heparin and Related Compounds, Brit. J. Exper. Path. **38**: 237, 1957.
11. Courtice, F. C., and Steinbeck, A. W.: Absorption of Protein From the Peritoneal Cavity, J. Physiol. **114**: 336, 1951.
12. Lake, B. J., Simmonds, W. J., and Steinbeck, A. W.: The Effect of Foreign Plasma on Capillary Permeability in Unsensitized Animals, Australian J. Exper. Biol. & M. Sc. **31**: 55, 1953.
13. Pillemer, L., Ratnoff, O. D., Blum, L., and Lepow, I. H.: The Inactivation of Complement and Its Components by Plasmin, J. Exper. Med. **97**: 573, 1953.
14. Burrows, W.: Jordan-Burrows Textbook of Bacteriology, Philadephia, 1950, W. B. Saunders Company.
15. Humphrey, J. H., and Jaques, R.: The Histamine and Serotonin Content of the Platelets and Polymorphonuclear Leucocytes of Various Species, J. Physiol. **124**: 305, 1954.
16. Benditt, E. P., Wong, R. L., Arase, M., and Roeper, E.: 5-Hydroxytryptamine in Mast Cells, Proc. Soc. Exper. Biol. & Med. **90**: 303, 1955.
17. Rowley, D. A., and Benditt, E. P.: 5-Hydroxytryptamine and Histamine as Mediators of the Vascular Injury Produced by Agents Which Damage Mast Cells in Rats, J. Exper. Med. **103**: 399, 1956.
18. Doepfner, W., and Cerletti, A.: Comparison of Lysergic Acid Derivatives and Antihistamines as Inhibitors of the Edema Provoked in the Rat's Paw by Serotonin, Internat. Arch. Allergy **12**: 89, 1958.
19. Miles, A. A., and Wilhelm, D. L.: Enzyme-Like Globulin From Serum Reproducing the Vascular Phenomena of Inflammation. I. An Activable Permeability Factor and Its Inhibitor in Guinea Pig Serum, Brit. J. Exper. Path. **36**: 71, 1955.
20. Stewart, P. B., and Bliss, J. Q.: The Permeability-Increasing Factor in Diluted Human Plasma, Brit. J. Exper. Path. **38**: 462, 1957.
21. Mill, P. J., Elder, J. M., Miles, A. A., and Wilhelm, D. L.: Enzyme-like Globulins From Serum Reproducing the Vascular Phenomena of Inflammation V. Activable Permeability Factor in Human Serum, Brit. J. Exper. Path. **39**: 335, 1958.
22. Boake, W. C., and Lovell, R. R. H.: The Necrotizing Activity of Human Sera for Guinea Pig Skin, Brit. J. Exper. Path. **35**: 350, 1954.
23. Kuroyanagi, T.: Studies on Capillary Permeability Promoting Action of Serum; Its Relations to Fibrinolysis and Increased Capillary Permeability, Tohoku J. Exper. Med. **62**: 353, 1955.
24. Wisseman, C. L., Jr., and Greisman, S. E.: Unpublished data.

EFFECTS OF CORTICOSTEROID ADMINISTRATION ON THE SERUM ELECTROPHORETIC PATTERNS OF PATIENTS WITH PULMONARY TUBERCULOSIS

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During the study of the electrophoretic patterns of proteins and glycoproteins of patients recovering from pulmonary tuberculosis, it was noticed that those who received corticosteroids as an adjunct to therapy exhibited a markedly different protein response than those on antituberculosis drugs alone. Before treatment the serum proteins were lowered in the albumin fraction with increases in the alpha$_2$ and gamma components and elevated glycoprotein levels as is usually seen in extensive exudative disease. Although comparable x-ray clearing occurred in the cortisone group, further increases in the alpha$_2$, beta, and gamma globulin fractions occurred, while in the group not treated with cortisone these values returned to normal. The glycoprotein components of the group treated with cortisone declined during this period, indicating that the increases were due to carbohydrate-poor proteins. A statistical analysis is included.

SERUM protein patterns in pathologic states in which the inflammatory response is a prominent facet are characterized by a decrease in the albumin fraction and increases in the protein and protein-bound carbohydrates of the globulin fractions.[1, 2] These changes have been studied extensively in tuberculosis and are further characterized by a rapid return to normal after treatment is instituted.[3, 4] In conditions benefited by the administration of corticosteroids, the serum protein patterns have also been observed to return to normal.[5, 6]

In the present investigation, serum samples from two groups of patients with extensive pulmonary tuberculosis have been analyzed by filter paper electrophoresis before and after 3 months of chemotherapy. The electrophoretic patterns of the group that received corticosteroids in addition to chemotherapeutic agents exhibited changes that were unexpected in view of the favorable treatment response of the patients.

METHODS

Clinical Appraisal.—Patients recently admitted were selected for the study on the basis of roentgenographic evidence of extensive early disease. Serum chemistry and x-ray

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findings were comparable in both groups prior to therapy. Group I received standard antituberculosis chemotherapy consisting of streptomycin, isoniazid, and para-aminosalicylic acid, in various combinations. Group II received the same treatment plus corticosteroids. Indications for corticosteroid therapy were either extensive, soft infiltrative disease that did not respond optimally after a short period of antituberculosis therapy, or toxicity manifested by daily fever, debilitation, etc. Although several adrenocortical steroids were used, prednisone was most commonly employed. Initial daily dosage ranged from 24 to 32 mg. At the time of the follow-up examination, the patients were receiving 8 to 16 mg. per day.

Electrophoretic Analyses.—Electrophoretic analyses were carried out by procedures previously reported.[4] Filter paper strips were stained for protein with bromphenol blue and for glycoprotein with the periodic acid–Schiff reagent. Absolute concentrations were determined by reference to total serum glycoprotein and total serum protein concentrations.[4] The mean, standard error of the mean, *t*, and probability values were computed by standard statistical methods.[7]

RESULTS

Both groups of patients showed a favorable response to the treatment regimens as indicated by disappearance of toxicity, resolution of x-ray infiltration, and weight gain. These objective findings often occurred more rapidly in the corticosteroid treated group.

TABLE I. EFFECTS OF CORTICOSTEROIDS ON SERUM PROTEIN LEVELS OF PATIENTS WITH PULMONARY TUBERCULOSIS

GROUP	NO. CASES	TOTAL SERUM PROTEIN* (GM./100 ML.)	ALBUMIN* (GM./100 ML.)	GLOBULINS (GM./100 ML.)			
				ALPHA$_1$*	ALPHA$_2$*	BETA*	GAMMA*
Normal	30	7.1 ± 0.20	4.9 ± 0.21	0.2 ± 0.04	0.4 ± 0.06	0.6 ± 0.04	1.0 ± 0.12
I, Pretreatment	16	6.4 ± 0.20†	3.2 ± 0.20‡	0.3 ± 0.09	0.7 ± 0.08‡	0.7 ± 0.07	1.5 ± 0.10‡
II, Pretreatment	11	6.9 ± 0.20	3.6 ± 0.30‡	0.3 ± 0.04	0.7 ± 0.07‡	0.7 ± 0.06	1.6 ± 0.24†
I, Posttreatment	16	7.2 ± 0.12	4.9 ± 0.12	0.2 ± 0.01	0.4 ± 0.01	0.6 ± 0.01	1.1 ± 0.03
II, Posttreatment	11	7.3 ± 0.29	3.1 ± 0.17‡	0.3 ± 0.02	0.9 ± 0.05‡	1.0 ± 0.06‡	2.0 ± 0.34‡

*Including the standard error of the mean.
†$p < 0.05 > 0.01$.
‡$p < 0.01$.

The results of the serum protein analyses are presented in Table I. Prior to treatment the serum patterns of both patient groups exhibited the decrease in albumin and increases in the alpha$_2$ and gamma globulins usually found in early, extensive exudative disease.[4] After 3 months' therapy pronounced differences in the serum protein patterns were observed. The pattern for Group I had returned to normal. Group II, although showing the same degree of clinical improvement, had an electrophoretic pattern that had diverged farther from normal. The alpha$_2$, beta, and gamma globulins had increased over pretherapy levels. The further decline in albumin concentration was not statistically significant.

Protein-bound carbohydrate values were significantly elevated in both groups of patients for total serum and the alpha$_1$ and alpha$_2$ globulin fractions before treatment (Table II). The serum glycoprotein concentrations of Group I reverted to the normal range after 3 months' chemotherapy. The protein-bound carbohydrate concentrations for total serum and the alpha$_1$ and alpha$_2$ globulin fractions of Group II also diminished during treatment. However, the bound carbohydrate of the alpha$_1$ globulin fraction was the only one of the previously elevated values to return to the normal level.

TABLE II. EFFECTS OF CORTICOSTEROIDS ON SERUM GLYCOPROTEIN* LEVELS OF PATIENTS WITH PULMONARY TUBERCULOSIS

GROUP	NO. CASES	TOTAL SERUM GLYCOPROTEIN† (MG./100 ML.)	ALBUMIN GLYCOPROTEIN† (MG./100 ML.)	GLOBULIN GLYCOPROTEINS (MG./100 ML.)			
				ALPHA$_1$†	ALPHA$_2$†	BETA†	GAMMA†
Normal	30	110 ± 6.1	16 ± 3.1	22 ± 2.0	26 ± 3.8	26 ± 2.5	20 ± 1.5
I, Pretreatment	16	201 ± 4.5‡	19 ± 2.5	37 ± 3.4‡	73 ± 7.8‡	45 ± 4.9‡	27 ± 5.1
II, Pretreatment	11	184 ± 1.3‡	13 ± 3.0	33 ± 3.1‡	74 ± 7.0‡	35 ± 4.5	29 ± 4.0
I, Posttreatment	16	111 ± 2.7	15 ± 1.5	23 ± 1.3	25 ± 1.1	27 ± 1.3	21 ± 1.5
II, Posttreatment	11	153 ± 8.0‡	22 ± 2.4	21 ± 2.1	53 ± 2.6‡	33 ± 2.9	24 ± 3.7

*The use of the term glycoprotein with respect to the serum fractions is not intended to imply that only one carbohydrate containing protein is present.
†Including the standard error of the mean.
‡$p < 0.01$.

Typical protein and glycoprotein patterns for all groups are illustrated in Fig. 1.

Decreased glycoprotein to protein ratios (glycoprotein/protein × 100) were found for the globulin fractions of Group II after therapy (Table III). The decreases were significant with respect to pretreatment ratios, and suggested that the increased protein concentrations of the alpha$_2$, beta, and gamma globulin fractions were due primarily to carbohydrate-poor proteins.

TABLE III. SUMMARY OF GLYCOPROTEIN TO PROTEIN RATIOS

GROUP	GLYCOPROTEIN : PROTEIN × 100 (%)					
	TOTAL SERUM*	ALBUMIN*	ALPHA$_1$ GLOBULIN*	ALPHA$_2$ GLOBULIN*	BETA GLOBULIN*	GAMMA GLOBULIN*
Normal	1.6 ± 0.08	0.3 ± 0.03	11.0 ± 1.42	6.5 ± 0.40	4.3 ± 0.38	2.0 ± 0.23
I, Pretreatment	3.1 ± 0.20†	0.6 ± 0.26	12.3 ± 2.66	10.4 ± 1.49‡	6.4 ± 0.60‡	1.8 ± 0.20
II, Pretreatment	2.7 ± 0.20†	0.4 ± 0.11	11.0 ± 0.53	10.6 ± 1.10‡	5.0 ± 0.80	1.8 ± 0.29
I, Posttreatment	1.5 ± 0.05	0.3 ± 0.03	11.5 ± 0.74	6.3 ± 0.34	4.5 ± 0.22	1.9 ± 0.16
II, Posttreatment	2.1 ± 0.14†	0.7 ± 0.29‡	7.0 ± 1.01†	5.9 ± 1.31	3.3 ± 0.23	1.2 ± 0.70†

*Including the standard error of the mean.
†$p < 0.01$.
‡$p < 0.05 > 0.01$.

Six patients each from Groups I and II were "matched" on the basis of the roentgenograph before treatment. The average chemical values for the matched cases were similar to the mean values for their respective groups both before and after treatment. The roentgenographs of Group II in general showed more resolution of the disease than did Group I after 3 months.

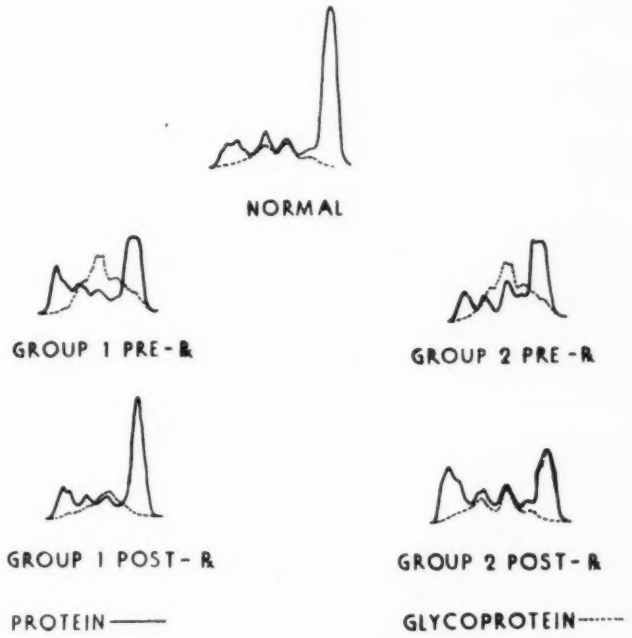

Fig. 1.—Typical electrophoretic patterns.

DISCUSSION

The effects of the administration of corticosteroids on the concentration and distribution of the proteins and glycoproteins of serum have been the subject of several clinical and experimental investigations. In pathologic states which are benefited by corticosteroid therapy, the administration of the hormone has resulted in both the protein and glycoprotein patterns returning to normal.[1, 2, 5, 6] These changes, however, probably reflect an indirect response to clinical improvement of the patients rather than a direct effect of the hormone therapy on the factors regulating serum protein levels.

The present study has demonstrated the diversity of the response of the protein-bound carbohydrates and proteins of serum in pulmonary tuberculosis after treatment with corticosteroids and with chemotherapeutic agents. It seems probable that alterations observed in the protein patterns of the patients who received antituberculosis agents only (Group I) reflected an indirect response to clinical improvement. The role of corticosteroids in the metabolism of the serum proteins has not been established. Roberts[8] has suggested that the primary role of the adrenal cortical secretions in protein metabolism is stimulation

of the mobilization of tissue protein. Clark[9] has proposed that the major effect may be a partial inhibition of serum protein anabolism. The observations made on the Group II patients give some support to Robert's hypothesis.

Increased serum glycoprotein concentrations have been observed in a wide variety of unrelated pathologic states.[2] It has been suggested that the elevated levels may represent a systemic response to nonspecific stress.[10] Although the mechanism is still speculative, it is generally accepted that adrenocortical secretions are concerned with the ability of the organism to respond to stress. However, the injection of cortisone into normal guinea pigs,[11] rats,[12] and rabbits[13] had no effect on the concentration of total serum glycoprotein or the carbohydrate-rich seromucoid fraction. Evidence has been reported which suggests that corticosteroids may exert a homeostatic effect on the serum glycoproteins.[11] The results obtained with the patients who received hormone therapy tend to support the concept.

Recent investigations on the development and application of the techniques of starch gel electrophoresis,[14] anion-exchange cellulose chromatography,[15] and immunoelectrophoresis[16] have provided substantial evidence for the heterogeneity of the classic fractions of the serum proteins. In view of the multiplicity of the protein components of serum, it seems probable that several different physiologic mechanisms may be operative in their metabolism. The present results are in accord with this premise.

The separation of tissue anabolism and catabolism in the intact organism presents formidable difficulties which as yet have not been resolved. It might be anticipated that the application of the more recent and sensitive techniques of protein fractionation cited would provide additional information on the influence of corticosteroid administration on the simple and conjugated proteins of serum.

SUMMARY

The partition of the serum proteins and glycoproteins has been investigated by filter paper electrophoresis in two groups of patients with pulmonary tuberculosis before and after different treatment regimens. One group received conventional antituberculosis therapy and the other corticosteroids in addition to routine chemotherapy. The clinical and x-ray findings were similar in both groups before treatment. Prior to treatment the serum protein patterns of both groups exhibited the decrease in albumin and increases in the $alpha_2$ and gamma globulin fractions and the elevated glycoprotein levels usually found in extensive exudative disease. After treatment the roentgenographs of the group treated with corticosteroids in general showed more resolution of the disease than the group treated with antibiotics alone. The serum protein and glycoprotein patterns in the group that had received only antimicrobial therapy had returned to normal after 3 months' treatment. In the group that received corticosteroids, further increases were observed in the $alpha_2$, beta, and gamma globulin fractions. A decline in the glycoprotein to protein ratios for the fractions indicated that the increase was due to carbohydrate-poor proteins. The results are discussed with respect to various physiologic mechanisms that may be involved.

REFERENCES

1. Wuhrman, F., and Wunderly, C.: Die Bluteiweisskörper des Menschen, Basel, 1957, Benno Schwabe & Co.
2. Winzler, R. J.: Determination of Serum Glycoproteins, *in* Glick, D., editor: Methods of Biochemical Analysis, New York, 1955, Interscience Publishers, Inc., vol. 2, p. 279.
3. Lawrence, S. H., Weimer, H. E., and Salkin, D.: Serial Serum Glycoprotein and Protein Studies in Pulmonary Tuberculosis, J. LAB. & CLIN. MED. **51**: 935, 1958.
4. Lawrence, S. H., Weimer, H. E., and Salkin, D.: Serial Electrophoretic Studies of the Serum Glycoproteins and Proteins in Patients With Pulmonary Tuberculosis, Clin. Chim. Acta **4**: 374, 1959.
5. Shetlar, M. R., Schmidt, H. L., Lincoln, R. B., Devore, J. D., Bullock, J. J., and Hellbaum, A. A.: Response of the Serum Polysaccharide Fractions and Protein Fractions Following Cortisone Treatment of Patients With Rheumatic Fever, J. LAB. & CLIN. MED. **39**: 372, 1952.
6. Sprague, R. G., Power, M. H., Mason, H. L., Albert, A., Mathieson, D. R., Hench, P., Kendall, E. C., Slocumb, C. H., and Palley, H. F.: Observations on the Physiologic Effects of Cortisone and ACTH in Man, Arch. Int. Med. **85**: 199, 1950.
7. Snedecor, G. W.: Statistical Methods, ed. 4, Ames, Iowa, 1946, Iowa State College Press.
8. Roberts, S.: The Influence of the Adrenal Cortex on the Mobilization of Tissue Proteins, J. Biol. Chem. **200**: 77, 1953.
9. Clark, I.: The Effect of Cortisone Upon Protein Synthesis, J. Biol. Chem. **200**: 69, 1953.
10. Boas, N. F., and Peterman, A. F.: Effects of Age, Food Intake, and Stress on Plasma Hexosamine Levels in the Rat, Proc. Soc. Exper. Biol. & Med. **82**: 19, 1953.
11. Weimer, H. E., and Redlich-Moshin, J.: Comparative Effects of Intra-muscular Injections of ACTH, Cortisone, and Saline on Serum Glycoprotein Levels, Proc. Soc. Exper. Biol. & Med. **84**: 34, 1953.
12. Shetlar, M. R., and Shetlar, C. L.: Effect of Cortisone on Serum Glycoprotein and Seromucoid Levels of Rats, Proc. Soc. Exper. Biol. & Med. **88**: 622, 1955.
13. Heppleston, A. G., and Keyser, J. W.: The Relationship of Tissue Formation and Destruction to the Serum Protein-Bound Carbohydrate Levels in Experimental Inflammation, Brit. J. Exper. Path. **38**: 111, 1957.
14. Poulik, M. D., and Smithies, O.: Comparison and Combination of the Starch-Gel and Filter Paper Electrophoretic Methods Applied to Human Sera: Two Dimensional Electrophoresis, Biochem. J. **68**: 636, 1958.
15. Fahey, J. L., McCoy, P. F., and Goulian, M.: Chromatography of Serum Proteins in Normal and Pathologic Sera: The Distribution of Protein-Bound Carbohydrate and Cholesterol, Siderophilin, Thyroxin-Binding Protein, B_{12} Binding Protein, Alkaline and Acid Phosphatases, Radio-iodinated Albumin and Myeloma Proteins, J. Clin. Invest. **37**: 272, 1958.
16. Grabar, P.: The Use of Immunochemical Methods in Studies on Proteins, *in* Glick, D., editor: Methods of Biochemical Analysis, New York, 1958, Interscience Publishers, Inc., vol. 13, p. 1.

COMPOSITION AND FUNCTION OF HUMAN SYNOVIAL CONNECTIVE TISSUE CELLS MEASURED IN VITRO

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To appreciate the multiple interrelationships among the cell types composing synovial tissue, it is necessary to obtain objective information concerning the composition and function of the various cellular components of normal and pathologic tissue. To this end, cell culture techniques were employed to isolate and study the cellular derivatives from eight normal and five pathologic human synovial specimens.

Measurements of the growth characteristics, carbohydrate metabolism, and capacity for mucopolysaccharide synthesis revealed little difference among cells derived from normal and pathologic tissue. It was apparent that the cultured cells retained sufficient "differentiation" to produce highly polymerized hyaluronic acid while nourished by a semisynthetic medium. This mucopolysaccharide was found primarily in the supernatant medium and was elaborated by the cells during all phases of the growth cycle. Comparative growth curve studies appear to offer a sensitive means for detecting differences between cell lines.

THE individual contributions of the different cellular constituents of connective tissue to the metabolism of the whole tissue are not well delineated. Study of the inflammatory process, using histologic techniques, has outlined the sequence of events visible in tissue sections. These methods permitted description of the acute vascular phase of inflammation and led to recognition of sequential cellular proliferation, mucopolysaccharide accumulation, and collagen fiber deposition as major events in the reparative (anabolic) phase of the inflammatory reaction. Biochemical alterations in connective tissue components have been studied during the neoformation of this tissue in response to wounding,[1] polyvinyl sponge implantation,[2, 3] and carrageenin injection.[4] In general, such studies illuminate and support the earlier microscopic observations.

Information concerning the composition, functional capacities, nutritional requirements, regulatory mechanisms, and interaction of the diverse cellular elements can be expected from the application of cell culture techniques. For example, the amino acid requirements[5, 6] and patterns of carbohydrate utilization[7] are known for certain kinds of cells. Mucopolysaccharide synthesis by mixed connective tissue cell populations is well documented,[8-10] and recent studies suggest that quantitative measurement of the rate of synthesis is feasible.[11]

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METHODS

In the present study, cells were derived from eight normal and five pathologic human synovial tissue specimens and examined with respect to rate of in vitro proliferation, rate and pattern of carbohydrate utilization, and rate of mucopolysaccharide synthesis. In four cell lines, measurement of gross chemical composition of the cells was undertaken, and in five cases the viscosity of the newly formed mucopolysaccharide was studied.

Synovial tissue was obtained from the knee by needle biopsy, open arthrotomy, at autopsy, or after amputation. A portion of each specimen was prepared for histologic examination and the remainder was cut into 1 by 2 by 2 mm. fragments for explantation in Earle T-15 or T-30 flasks. Explants were placed directly on the glass surface under a layer of perforated cellophane. Nutrient medium was composed of 80 per cent CMRL medium 1066* and 20 per cent heat inactivated pooled human serum, supplemented with L-glutamine, penicillin, and streptomycin. Serum was inactivated by heating at 56° C. for 30 minutes, and then sterilized by filtration through a No. 6 Seitz pad at 6° C. to permit partial removal of serum lipids. Cultures were incubated at 37° C. and medium changes were performed at 2 to 3 day intervals. Used media were frozen for subsequent chemical analysis.

Sufficient primary outgrowth to permit subculturing usually occurred within 21 days of explantation. Cells were released from the glass around the original explants by incubation with 0.25 per cent trypsin† at 37° C. for 5 to 6 minutes. The resulting cell suspension was centrifuged at 800 r.p.m. for 5 to 7 minutes, the trypsin solution discarded, and the cells resuspended in fresh medium. Such a cell suspension is suitable for enumeration in hemocytometer, after which daughter cultures may be initiated directly on glass with a known number of cells. The packed cell volume of a known number of cells is determined by centrifugation in a Hopkins tube at 2,500 r.p.m. for 5 minutes. Sequential subcultures have been carried out at 5 to 10 day intervals for approximately 3 to 6 months after reaching the monolayer culture stage. Stock cultures are carried in Earle T-60 flasks by inoculating with 0.3×10^6 to 0.4×10^6 cells in 10 ml. of nutrient medium. After 6 to 8 days in culture, with medium changes at 2 to 3 day intervals, 2.0×10^6 to 4.0×10^6 cells may be harvested.

Hexose in culture media and cell extracts was measured by the anthrone method.[12] Lactic acid was determined by the Barker-Summerson method,[13] and cellular protein was measured by a modification of the procedure described by Lowry.[14] Routine measurement of medium hyaluronic acid, measured as uronic acid, was carried out by the euglobulin precipitation method described by Bollet.[15] Addition of 0.1 ml. 0.2M Na_2SO_4 prior to the final protamine precipitation improved recovery of hyaluronate added to culture media, usually yielding 70 to 80 per cent recovery. In some cases pepsin-trypsin deproteinization was used in place of the euglobulin precipitation technique, as a check on the latter method. In this procedure, 1 mg. of pepsin was added to 10 ml. of sample and dialyzed 18 to 24 hours against 0.1N HCl. After neutralizing the contents of the bag by dialysis against 1 per cent $NaHCO_3$, 1 mg. trypsin was added and dialysis continued for 2 days against 1 per cent $NaHCO_3$. Both enzyme digestions were carried out at 37° C., and at their conclusion residual protein was precipitated with perchloric acid at a final concentration of 4 per cent. Perchloric acid was removed by dialysis and the mucopolysaccharide precipitated with protamine as in the Bollet procedure. Recovery of added hyaluronate ranged from 70 to 85 per cent. The mucin clot forming and turbidigenic properties of used culture media were tested by addition of 0.13 ml. 7N acetic acid to 5.0 ml. of sample. The hyaluronidase lability of such acid-generated turbidity or mucin clots was occasionally verified with ribonuclease-free hyaluronidase.

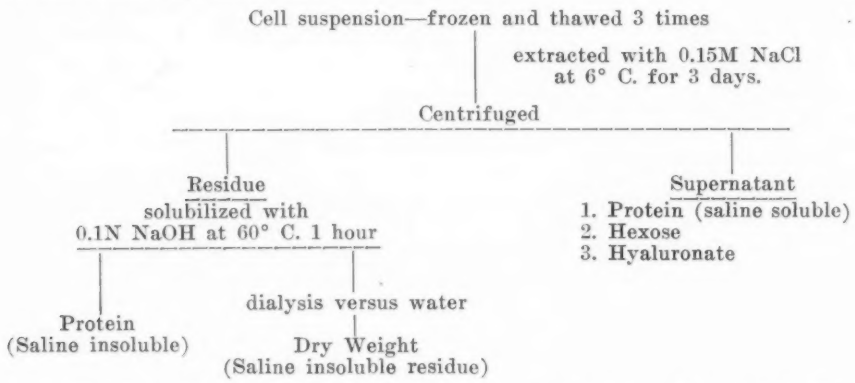
Electrodialysis is a satisfactory technique for concentrating and isolating "undegraded" mucopolysaccharide from synovial fluid.[16] Used medium was dialyzed against

*Connaught Medical Research Laboratory.
†Bacto-trypsin 1:250 (Difco).

a large volume of 0.03M phosphate buffer, pH 7.0, for 2 to 4 days and then concentrated to one fourth original volume by dialysis against polyvinylpyrrolidone. Electrodialysis of this material was performed in an apparatus with a capacity of approximately 10 ml.* The mucopolysaccharide deposits on the anodal side of the dialysis bag as a gel which can be scraped off, redissolved, and further purified by repeated electrodialysis. In the present experiments, electrodialysis was performed twice and the second gel was dissolved in water for study. The hyaluronate and protein concentrations of these preparations were measured and the relative viscosity was determined in an Ostwald viscosimeter at 37.2° C. Viscosimeters used ranged in size from 0.5 to 2.0 ml. and exhibited water flow rates between 6.5 and 61.5 seconds. Measurement of hyaluronate, as uronic acid, was carried out on a trichloroacetic acid supernatant after an approximately 18 hour incubation of sample with testicular hyaluronidase, at 37° C., in 0.1M acetate buffer, pH 5.8.

RESULTS

Protein, Hexose, and Hyaluronate Content of Synovial Connective Tissue Cells.—Suspensions of cells were washed twice with 0.15M NaCl and submitted to the following procedure:


Cell suspension—frozen and thawed 3 times
extracted with 0.15M NaCl at 6° C. for 3 days.
Centrifuged
Residue solubilized with 0.1N NaOH at 60° C. 1 hour
Supernatant
1. Protein (saline soluble)
2. Hexose
3. Hyaluronate
dialysis versus water
Protein (Saline insoluble)
Dry Weight (Saline insoluble residue)

The data derived from this procedure are presented in Table I. Approximately one half of the total cell protein was easily extractable with isotonic saline. A significant portion of the insoluble residue was a nondialyzable, nonprotein substance. Hexose was found in the saline soluble fraction in small

TABLE I. COMPOSITION OF WASHED SYNOVIAL CONNECTIVE TISSUE CELLS

DONOR	TOTAL PROTEIN (MG.)	SALINE SOLUBLE PROTEIN (MG.)	SALINE INSOLUBLE PROTEIN (MG.)	NONPROTEIN SALINE INSOLUBLE RESIDUE (MG.)	HEXOSE (MG.)	HYALURONATE (μG)
W. H.$_4$	11.54	5.39	6.15	5.15	0.445	13.0
J. S.	9.04	4.90	4.14	3.10	0.345	6.0
J. D.	4.69	2.32	2.37	3.63	0.140	15.0
M. K.*	19.87	12.10	7.77	21.43	0.610	22.0

*Pooled, washed "MK" cells were also subjected to grinding in a glass tissue grinder while frozen.

amounts, equivalent to 5 to 8 per cent of the saline soluble protein fraction. Of special interest was the demonstration of minor amounts of cell associated, nondialyzable hyaluronic acid. These studies suggest that 1 to 3 μg of *cell associated* hyaluronate may be found with each milligram of total cellular protein,

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whereas 20 to 100 μg of hyaluronate are found *in the culture medium* for each milligram of total cell protein. In other experiments, uronic acid determinations after pepsin-trypsin digestion of whole cells also indicated that the quantity of cell-associated hyaluronate was trivial in comparison with that found in the medium. The methods used here detect nondialyzable hyaluronic acid and thus provide no information concerning the presence of oligosaccharides in the cells and supernatant medium.

Rate of Proliferation.—Cell proliferation among the thirteen synovial cell lines was compared in terms of average generation time. Average generation time, defined as the mean time required for a cell population to double, was calculated from the following expressions:

$$\text{Number of cell divisions} = \frac{\log_{10}(\text{final cell population}) - \log_{10}(\text{inoculum})}{\log_{10}2}$$

$$\text{Average generation time} = \frac{\text{duration of culture (hr.)}}{\text{number of cell divisions}}$$

Since in old, nondividing cultures, the average value would be erroneously weighted toward a long generation time, we used only cultures of 5 to 10 days' duration to calculate the time required for a cell population to double. From Table II it is apparent that the average generation time of cells derived from normal tissue ranged from 47 to 100 hours, whereas the values for derivatives of abnormal tissue varied from 38 to 258 hours. Inspection of ranges of average generation time of the individual cell lines indicates that proliferative rate, measured in this way, is not related to presence or absence of pathologic conditions in the donor tissue or to the age or sex of the donors.

Rate of Hyaluronic Acid Production.—Since virtually all of the mucopolysaccharide formed by the cultured synovial cells can be found in the supernatant medium, it is possible to obtain an idea concerning its rate of formation by use of the following expression:

$$\text{Rate of hyaluronic acid (HA) synthesis } (10^{-12}\ \text{Gm./cell/day}) = \frac{\text{total HA } (\mu g)}{\text{number of cells} \times \text{time (days)}}$$

Total medium hyaluronic acid refers to that amount synthesized after the final medium change, whereas the time factor refers to the number of days that the last medium change was in contact with the metabolizing cell mass. Use of the cell count determined at the end of the culture period may be justified in view of the relatively small change in cell numbers which occurs late in the growth cycle of a culture.

Data tabulated in Table II indicate that in the majority of the cell lines studied hyaluronate was formed at a mean rate of 10 to 20 picograms* per cell per day. The range of values obtained with the individual cell lines, as well as the mean rates of hyaluronate synthesis, is similar despite variations in the age, sex, and health of the tissue donors.

*A picogram (pg.) equals 10^{-12} Gm.

TABLE II. RATES OF PROLIFERATION AND HYALURONATE SYNTHESIS MEASURED IN SYNOVIAL CELLS OF NORMAL AND PATHOLOGIC ORIGIN

DONOR	SEX	AGE	SYNOVIAL PATHOLOGY	MONTHS CULTURED	NO. OF SUBCULTURES	AVERAGE GENERATION TIME (HR.)*			HYALURONIC ACID 10^{-12} GM./CELL/DAY		
						NO. OBS.	MEAN	RANGE	NO. OBS.	MEAN	RANGE
D. S.	M	11	None	5	19	18	66	39-94	5	36	10-55
J. S.	F	16	None (SLE)†	4	14	8	47	34-49	7	8	2-20
D. G.	M	29	None	4	11	5	96	56-163	6	7	2-21
E. E.	M	39	None	4	15	13	88	29-127	4	12	6-21
W. H.$_4$	M	50	None	9	26	11	68	35-94	13	11	3-37
W. J.	M	57	None	5	17	17	78	53-124	8	25	9-46
P. D.	M	69	None	5	18	8	100	55-160	10	35	10-54
M. K.	F	73	None	4	13	21	69	31-100	20	17	6-47
W. H.	M	20	PVS‡	4	14	10	93	38-154	2	22	10-34
R. B.	M	33	Tr. S.§	6	14	3	85	61-99	6	22	9-35
J. D.	M	42	Rheum. Arth.	4	13	7	62	43-67	8	17	9-24
R. V. W.	M	43	Rheum. Arth.	2	6	2	59	52-66	1	29	—
K. E.	F	53	Rheum. Arth.	3	6	4	191	45-258	2	45	43-48

*Average generation time is the time required for a cell population to double.
†S. L. E. = Systemic lupus erythematosis.
‡PVS = pigmented villonodular synovitis.
§Tr. S. = Traumatic synovitis.

Viscous Property of Culture-Produced Hyaluronic Acid.—During in vitro propagation of these cell lines, five began to elaborate into the supernatant medium mucopolysaccharide which gave a positive mucin clot test. We have recognized that synthetic media supplemented only with human serum will support hyaluronate synthesis sufficiently to permit turbidity formation on acidification of the used culture medium. The demonstration of frank mucin clots was a new development. Incubation of medium with testicular hyaluronidase destroyed the clot forming property. A surprising aspect of this observation was the fact that the clot forming property developed *after* cell lines had been in serial subculture for varying lengths of time. Mucin clot formation was detectable in "WH_4" after the eighth passage, in "MK" after the seventh subculture, in "JS" after the first passage, in "JD" after the eighth subculture, and in "CO"* after the third transfer. Of equal interest was the relatively low concentration of hyaluronate in medium with the clotting property. Both the euglobulin and pepsin-trypsin methods yielded values of 7 to 23 μg of uronic acid-containing mucopolysaccharide per milliliter of medium. In the case of the "CO" cell line, agitated shake cultures as well as the conventional stationary glass substrate cultures yielded media with the clotting property. Indeed, media from the shake culture gave our most impressive mucin clots.

Pooled media from each of the five cell lines carried in stationary culture, as well as medium from the shake culture experiment, were subjected to electrodialysis in the fashion indicated earlier. The relative viscosity, hyaluronate concentration, and protein concentration are recorded in Table III.

TABLE III. RELATIVE VISCOSITY OF HYALURONATE PRODUCED BY SYNOVIAL CELLS IN VITRO

DONOR	RELATIVE VISCOSITY	HYALURONATE CONCENTRATION (MG./ML.)	PROTEIN CONCENTRATION (MG./ML.)
W. $H._4$	4.6	0.298	0.74
C. O. (shake)	8.0	0.460	1.00
C. O. (stationary)	4.6	0.685	7.50
J. S.	3.4	0.538	4.20
J. D.	3.2	0.384	3.60
M. K.	1.5	0.121	2.65
Water	1.0	0.00	0.00
Medium (unused)	1.16	0.00	15.00
Serum	1.6	0.00	75.00

Since the hyaluronate gels were electrodeposited from a solution of 0.03M phosphate buffer, pH 7.0, and subjected to study after dilution in distilled water, the salt concentration would have varied from 0.003M to 0.01M among the various samples. Whereas the viscous nature of the isolated material is apparent, small variations in salt concentrations will alter relative viscosity measurements and render strict comparison of the viscosity values untenable.

In order to compare the viscous properties of hyaluronate derived from different sources, samples were adjusted by dialysis to pH 8.1 in a buffer which was 0.1M with respect to both NaCl and $NaHCO_3$. As a standard for comparison, hyaluronate was isolated by electrodialysis from joint fluid aspirated

*Synovial cell line from normal joint tissue of 42-year-old woman with systemic lupus erythematosus.

from a patient with rheumatoid arthritis. Fig. 1 illustrates the linear relationship between relative viscosity (ηrel) and hyaluronate concentration at low concentrations of this standard material.

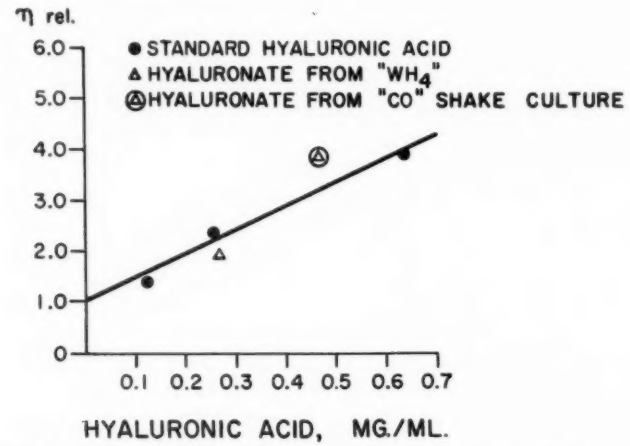

Fig. 1.—Relative viscosity of hyaluronic acid solutions plotted against solute concentration.

It is apparent that viscosity-concentration data from culture-produced hyaluronate fall close to the line representing the viscous behavior of the standard (produced in vivo) hyaluronic acid. Calculation of intrinsic viscosity [η] as proposed by Sundblad[17]* yields values ranging from 32 to 54 for the standard hyaluronate and values of 38 and 62 for hyaluronate produced by "WH_4" and "CO," respectively. After adjustment of pH to 5.8 by dialysis against 0.05M acetate buffer, the relative viscosity was 1.7 and 3.4 units for "WH_4" and "CO" hyaluronate solutions, respectively. Addition of 0.05 ml. of testicular hyaluronidase solution† to 1.0 ml. of sample caused a prompt fall in the relative viscosity of each sample to 1.0.

Cell Volume and Protein Content.—Mean cell volume, determined from packed cell volume and the total cell count, ranged from 4,000 to 9,000 cubic microns among the various cell lines. The variation within individual cell lines was of the same order of magnitude (Table IV). There is a rough correlation between cell volume and proliferative rate, more rapidly dividing cells tending to be smaller. Mean cellular protein varied with cell volume and protoplasmic concentration of protein. Protein concentration was somewhat less variable than other parameters studied, most values falling between 50 and 70 μg of protein per microliter of packed cell volume. Whereas variations in cell size and protein content appear unrelated to donor age, the data do not warrant conclusions regarding the effect of donor sex or the presence of pathologic conditions in the original tissue.

Carbohydrate Utilization.—Hexose (glucose) consumption, measured over the last 2 to 3 days of the culture growth cycle, was related to total cellular

*$[\eta] = \frac{\eta sp}{c}$; where c = concentration of hyaluronic acid in grams per 100 ml.

†10,000 TRU of ribonuclease-free testicular hyaluronidase per 0.05 ml. solution.

TABLE IV. CELL VOLUME AND PROTEIN MEASURED IN SYNOVIAL CELLS OF NORMAL AND PATHOLOGIC ORIGIN

DONOR	SYNOVIAL PATHOLOGY	MEAN CELL VOLUME (CUBIC MICRONS) NO. OBS.	MEAN	RANGE	MEAN CELL PROTEIN (10^{-12} GM./CELL) NO. OBS.	MEAN	RANGE	PROTEIN CONCENTRATION ($\mu G/\mu L$ PCV) NO. OBS.	MEAN	RANGE
P. D.	None	2	11,575	8,050-15,100	1	840	—	1	56	—
W. J.	None	6	8,700	5,170-13,050	5	570	264-770	5	65	51-74
E. E.	None	4	8,010	4,600-10,900	2	495	340-650	2	65	57-74
D. S.	None	6	8,045	5,130-10,700	3	407	343-520	3	56	53-83
W. H_4	None	10	5,132	2,857- 8,800	10	305	127-696	10	59	25-81
M. K.	None	9	5,600	3,550- 6,800	9	385	270-490	9	70	61-102
J. S.	None (SLE)	4	3,870	2,720- 4,950	4	320	288-348	4	88	60-127
J. D.	Rheum. Arth.	3	5,050	4,330- 5,920	3	394	364-428	3	78	72-84

protein in order to obtain a measure of uptake rate. As shown in Table V, the hexose consumption usually varied from 2.0 to 5.0 mg. of hexose per milligram of cell protein per day. The rate of hexose uptake was not clearly related to the proliferative rate.

Since it is likely that the lactic acid and hyaluronic acid which accumulate in the medium are derived from the hexose (glucose) taken up by the cells, the fraction of hexose uptake which can be accounted for by these products was calculated. Most of the hexose consumed (57 to 93 per cent) is converted to lactic acid, whereas only 1 to 2 per cent can be accounted for as hyaluronic acid.

Comment.—The proliferative rate and rate of hyaluronic acid synthesis, as presented above, reflect the "average performance" of a culture over its entire span as an integrated entity. On the other hand, the comparative data on rate and mode of carbohydrate utilization reflect the events of the latter 2 or 3 days of the culture span. In an effort to study these rate phenomena during the growth cycle of a culture, growth curve studies were performed.

Growth curve studies: Growth curve studies were set up using monolayer cultures of cell line "DS" (fifteenth passage) and cell line "WH_4" (fifth passage). In both cases, 12 T-60 flasks were inoculated with 0.4×10^6 cells each and incubated in the usual manner. The experiments differed in that "DS" was supplied with glucose at a concentration of 1.0 mg. per milliliter, while "WH_4" received 2.0 mg. per milliliter. On the second, fourth, sixth, and eighth days, 3 vessels of each cell line were sacrificed and the cellular and medium

TABLE V. CARBOHYDRATE UTILIZATION OF SYNOVIAL CELLS OF NORMAL AND PATHOLOGIC ORIGIN

DONOR	SYNOVIAL PATHOLOGY	HEXOSE CONSUMED (MG. HEXOSE/MG. PROTEIN/DAY) NO. OBS.	MEAN	RANGE	$\frac{\text{LACTIC ACID FORMED}}{\text{HEXOSE CONSUMED}} \times 100$ NO. OBS.	MEAN (%)	RANGE (%)	$\frac{\text{HYALURONATE FORMED}}{\text{HEXOSE CONSUMED}} \times 100$ NO. OBS.	MEAN (%)	RANGE (%)
P. D.	None	1	3.86	—	1	64	—	2	1.06	0.87-1.26
W. J.	None	5	2.23	1.70-3.20	1	57	—	7	1.52	0.86-2.83
E. E.	None	2	4.70	4.00-5.40	1	58	—	2	0.67	0.55-0.80
D. S.	None	1	12.00	—	1	56	—	3	0.89	0.7 -1.06
W. H_4	None	3	3.20	2.66-3.85	4	81	35-107	2	1.3	1.05-1.54
M. K.	None	8	3.02	2.40-4.42	11	93	63-118	13	1.56	0.39-4.55
J. S.	None (SLE)	4	1.95	0.83-4.04	8	84	65-105	7	1.51	0.38-3.40
J. D.	Rheum. Arth.	3	1.94	1.62-2.31	9	84	57-114	9	1.90	0.92-2.83

components were analyzed. Medium was changed in the remaining vessels not yet sacrificed at these intervals. Measurements of the cellular system, recorded as total cell count, packed cell volume, and total culture protein, were thus performed in triplicate at each point of both growth curves. However, for those measurements made of medium components, as hexose, lactic acid, and hyaluronic acid, twelve measurements were available at 2 days, nine for the 2 to 4 day interval, six for the 4 to 6 day interval, and three for the final 2 day period of the study.

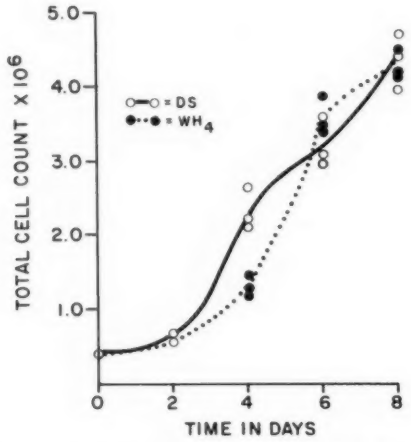

Fig. 2.—Increase in cell number during the growth cycle.

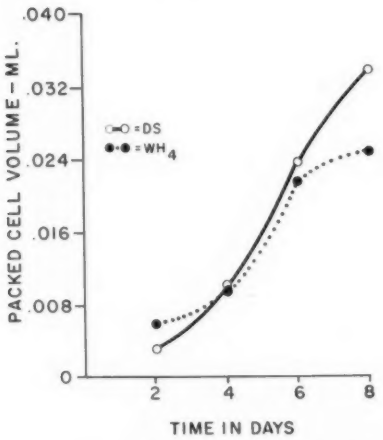

Fig. 3.—Increase in packed cell volume during the growth cycle.

Behavior of cell mass during growth cycle: Inspection of Fig. 2 reveals that an approximately tenfold increase in cell numbers occurred over the 8 day growth period. Each cell line showed an initial lag phase followed by accelerated growth, most rapid during the 2 to 4 day interval for "DS" and during the subsequent 2 day interval for "WH_4." Although the proliferative cycle was similar with respect to cell number, Fig. 3 reveals that "WH_4" did not

accumulate protoplasmic mass as rapidly as did "DS," presumably because of production of smaller cells. Even greater divergence in synthetic performance is seen in total culture protein measurements (Fig. 4). Some pertinent rate calculations are summarized in Table VI. While the over-all increase in cell numbers was similar for the two cell lines, the average generation time measurements reveal differing rates of proliferation at all phases of the growth cycle, including the period of logarithmic growth. Cells of the "DS" line tended to increase in size as the culture aged, while those of "WH_4" became smaller. Similarly, while the protein concentration of cell line "DS" became dilute with culture maturation, the reverse tendency is apparent in the "WH_4" experiment.

Carbohydrate utilization during the growth cycle: Medium glucose is consumed with increasing speed as the cell mass grows (Fig. 5). With both cell lines, 80 to 90 per cent of the hexose consumed can be accounted for as lactic acid in the medium. Where the higher medium glucose concentration

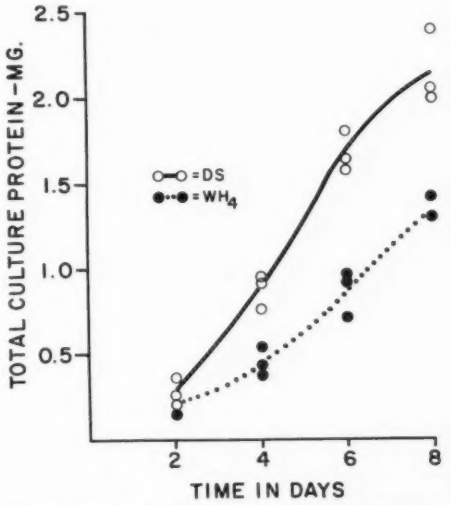

Fig. 4.—Increase in total culture protein during the growth cycle.

TABLE VI. CHANGES IN CELLULAR PARAMETERS DURING THE GROWTH CYCLE

PARAMETERS MEASURED	"DS" LINE GROWTH CYCLE INTERVALS (DAYS)				"WH_4" LINE GROWTH CYCLE INTERVALS (DAYS)			
	0-2	2-4	4-6	6-8	0-2	2-4	4-6	6-8
Average generation time (hr.)	79	25	107	107	90	40	33	200
Mean cell volume (μ^3)	5,400	4,680	7,450	7,750	10,900	7,600	6,000	5,840
Protein concentration ($\mu g/\mu l$)	81	81	70	63	36	45	40	54
Mean cell protein (Pg./cell)	443	373	529	491	390	339	238	314
Rate of H. A. synthesis (10^{-12} Gm./cell/day)	7	8	14	5	38	23	9	7
Rate of hexose consumption (mg. hexose/mg. protein/day)	4.40	3.80	2.71	1.97	12.00	10.40	8.90	5.60
Per cent hexose converted to lactic acid	93%	103%	86%	84%	77%	84%	94%	87%
Per cent hexose accounted for as hyaluronic acid	0.33%	0.57%	0.97%	0.55%	0.82%	0.64%	0.41%	0.37%

was present ("WH_4"), markedly increased glucose uptake and conversion to lactate occurred, without stimulation of growth in comparison with cell line "DS." In both cell lines, as the cultures aged, the rate of hexose consumption per unit of cell protein fell off in a manner roughly linear with time.

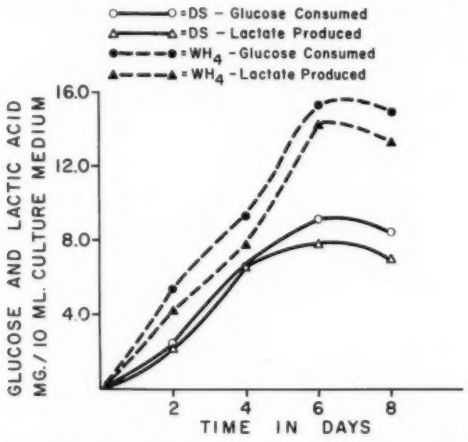

Fig. 5.—Carbohydrate utilization during the growth cycle.

Hyaluronic acid synthesis during the growth cycle: Measurement of medium hyaluronic acid revealed the presence of mucopolysaccharide during all portions of the growth cycle. Cumulative hyaluronate synthesis, plotted against time, resembles the curves for cell numbers, mass, and total culture protein (Fig. 6). The rate of mucopolysaccharide formation (see Table VI) changes

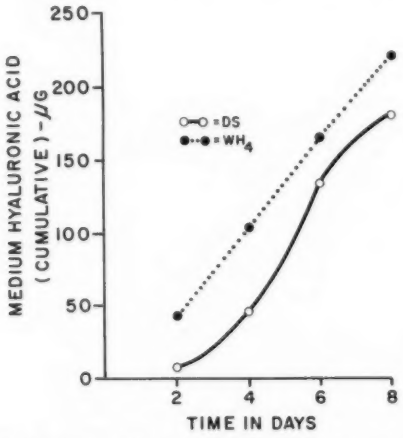

Fig. 6.—Hyaluronic acid synthesis during the growth cycle.

during the growth cycle, but is not clearly related to proliferative rate. Of particular interest is the tendency in "WH_4" for the rate of mucopolysaccharide synthesis to fall off in a linear fashion over the first 6 days of the culture. The data suggest the possibility of at least two cell populations, one which produced hyaluronate at a constant rate, but did not multiply, the second being primarily responsible for the increase in the cellular mass.

DISCUSSION

The data make it clear that cell cultures can be derived from normal and pathologic adult human tissues in a routine manner and propagated long enough to permit observation and experimental manipulation. Quantitative measurements of cell numbers, volume, protein content, and concentration, as well as estimates of the rates of glucose uptake and production of lactate and hyaluronate, appear similar in the various synovial cellular derivatives, irrespective of donor age, sex, or local pathologic condition in tissue.

It would be premature, however, to exonerate the connective tissue cells from suspicion of fundamental abnormality in the rheumatic diseases on the basis of the present evidence. The possibility that the similar "average performance" of the thirteen cell lines studied might have been imposed by the artificial environment requires further exploration. Whereas data based on the "average performance" of cultures disclosed few real differences, the growth curve technique yields more detailed information concerning culture dynamics and should add a new dimension to this type of study. Indeed, several parameters in the growth curves reported here behaved in opposite fashion in these two "normal" cell lines.

Growth curve studies with the L-strain mouse fibroblast indicate that glucose uptake per cell per unit time decreases as the culture ages, in a system where glucose levels were not replenished by medium renewal.[18] In the "non-replenished" system there was no proportionality between glucose uptake per cell and glucose concentration in the medium, which suggests that factors other than concentration of glucose governed its rate of uptake by the cell. In our studies with cells freshly derived from human connective tissue, the same time dependent decrease in rate of hexose uptake per unit of cell protein was observed in a system where periodic medium replacement assured adequate medium glucose levels.

It would be reassuring to know that in vitro cellular derivatives were unaltered from their in vivo state with respect to biochemical and physiologic properties. Although this degree of sophistication awaits realization, serial evaluation of chromosome numbers and structure is one qualitative control procedure which could be applied. To this end, "WH_4," "MK," "JS," and "JD" were examined with respect to chromosome number by the method of Tjio and Puck.[19] Although variation from the human diploid number (46) occurred as early as the second subculture, it was pertinent that at all stages of subculture studied, including the twentieth passage of "WH_4," the normal human diploid chromosome number was the one most frequently encountered.[20]

Previous reports have suggested that primary explant cultures of synovial tissue increase medium viscosity by addition of a mucopolysaccharide.[8, 21] In both reports measurements of relative viscosity were performed on the whole medium, and changes of the order of 0.05 to 0.2 relative viscosity units were recorded. Whereas the data provide qualitative evidence for the presence of a viscous mucopolysaccharide in the used culture medium, the intrinsic viscosity measurements require confirmation under circumstances less sensitive to minor observational error. In the present study, it was possible to isolate

sufficient quantities of mucopolysaccharide so that samples with relative viscosities ranging from 1.5 to 8.0 could be examined. Use of hyaluronate from rheumatoid joint fluid as a reference standard seems justified in view of recent work with whole fluid which indicates that the molecular weight of hyaluronic acid in normal and rheumatoid joint fluid is similar.[22] Since the intrinsic viscosity of culture produced and in vivo produced hyaluronate were similar, it is apparent that as least some synovial cells are able to synthesize high molecular weight hyaluronate after long periods in vitro on a semisynthetic diet. The primarily extracellular localization of highly polymerized hyaluronate in the cell culture system is in agreement with observations made on fresh synovial tissue using frozen section[23] and freeze-drying techniques.[24]

SUMMARY

1. A study of parameters of growth, carbohydrate metabolism, and synthetic capacity revealed little difference among cells derived from eight normal and five pathologic specimens when measured under circumstances which reflect either "average culture performance" or performance during the terminal phase of the growth cycle.

2. Comparative growth curve studies provide a more sensitive means for detecting differences between cell lines.

3. Hyaluronic acid of relatively high molecular weight is elaborated by synovial connective tissue cells nourished by a semisynthetic medium. Nearly all the highly polymerized hyaluronate formed by cultured cells is found in the medium, minor amounts being associated with the cells. This mucopolysaccharide is produced during all phases of the growth cycle, including the phase of logarithmic growth when glycolysis appears to be the important energy source.

REFERENCES

1. Dunphy, J. E., and Udupa, K. N.: Chemical and Histochemical Sequences in the Normal Healing of Wounds, New England J. Med. **253**: 847, 1955.
2. Boucek, R. J., and Noble, N. L.: Connective Tissue, a Technique for Its Isolation and Study, A. M. A. Arch. Path. **59**: 553, 1955.
3. Bollet, A. J., Goodwin, J. F., Simpson, W. F., and Anderson, D. V.: Mucopolysaccharide, Protein and Desoxyribonucleic Acid Concentration of Granulation Tissue Induced by Polyvinyl Sponges, Proc. Soc. Exper. Biol. & Med. **99**: 418, 1958.
4. Robertson, W. van B., and Schwartz, B.: Ascorbic Acid and the Formation of Collagen, J. Biol. Chem. **201**: 689, 1953.
5. Eagle, H.: Specific Amino Acid Requirements of Mammalian Cell (Strain L) in Tissue Culture, J. Biol. Chem. **214**: 839, 1955.
6. Harris, H., and Jahnz, M.: The Synthesis of Protein in the Rat Connective Tissue Cell, Brit. J. Exper. Path. **38**: 525, 1957.
7. Eagle, H., Barban, S., Levy, M., and Schulz, H. O.: The Utilization of Carbohydrates by Human Cell Cultures, J. Biol. Chem. **233**: 551, 1958.
8. Kling, D. H., Levine, M. G., and Weiss, J.: Mucopolysaccharides in Tissue Cultures of Human and Mammalian Synovial Membrane, Proc. Soc. Exper. Biol. & Med. **89**: 261, 1955.
9. Grossfeld, H., Meyer, K., Godman, G., and Linker, A.: Mucopolysaccharides Produced in Tissue Culture, J. Biophys. & Biochem. Cytol. **3**: 391, 1957.
10. Castor, C. W.: Production of Mucopolysaccharides by Synovial Cells in a Simplified Tissue Culture Medium, Proc. Soc. Exper. Biol. & Med. **98**: 80, 1957.
11. Castor, C. W.: The Rate of Hyaluronic Acid Production by Human Synovial Cells Studied in Tissue Culture, Arthritis & Rheumat. **2**: 259, 1959.

12. Seifter, S., Dayton, S., Novic, B., and Muntwyler, E.: The Estimation of Glycogen With the Anthrone Reagent, Arch. Biochem. **25:** 191, 1950.
13. Barker, S. B., and Summerson, W. H.: The Colorimetric Determination of Lactic Acid in Biological Material, J. Biol. Chem. **138:** 535, 1941.
14. Oyama, V. I., and Eagle, H.: Measurement of Cell Growth in Tissue Culture With a Phenol Reagent (Folin-Ciocalteau), Proc. Soc. Exper. Biol. & Med. **91:** 305, 1956.
15. Bollet, A. J., Seraydarian, M. W., and Simpson, W. F.: Acid Mucopolysaccharide in Normal Serum, J. Clin. Invest. **36:** 1328, 1957.
16. Roseman, S., Watson, D. R., Duff, I. F., and Robinson, W. D.: Electroconvection of Synovial Fluid, Fed. Proc. **14:** 878A, 1955.
17. Sundblad, L.: Studies on Hyaluronic Acid in Synovial Fluids, Acta Soc. med. upsal. **58:** 113, 1953.
18. Munyon, W. H., and Merchant, D. J.: The Relation Between Glucose Utilization, Lactic Acid Production and Utilization and the Growth Cycle of L Strain Fibroblasts, Exper. Cell Res. **17:** 490, 1959.
19. Tjio, J. H., and Puck, T. T.: Genetics of Somatic Mammalian Cells. II. Chromosomal Constitution of Cells in Tissue Culture, J. Exper. Med. **108:** 259, 1958.
20. Castor, C. W.: Chromosome Numbers in Cells Cultured From Human Synovial Tissue, Arthritis & Rheumat. **3:** 436, 1960.
21. Hedberg, H., and Moritz, V.: Biosynthesis of Hyaluronic Acid in Tissue Cultures of Human Synovial Membrane, Proc. Soc. Exper. Biol. & Med. **98:** 80, 1958.
22. Hamerman, D., and Schuster, H.: Synovial Fluid Hyaluronate in Rheumatoid Arthritis, Arthritis & Rheumat. **1:** 523, 1958.
23. Hamerman, D., and Ruskin, J.: Histologic Studies on Human Synovial Membrane. I. Metachromatic Staining and the Effects of Streptococcal Hyaluronidase, Arthritis & Rheumat. **2:** 546, 1959.
24. Castor, C. W.: The Microscopic Structure of Normal Human Synovial Tissue, Arthritis & Rheumat. **3:** 140, 1960.

ABNORMALITIES IN SERUM PROTEINS AND PROTEIN-BOUND HEXOSE IN HODGKIN'S DISEASE

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BETHESDA, MD.

The distribution of protein-bound hexose among serum globulin subfractions was investigated in Hodgkin's disease, in which total serum protein-bound hexose is frequently very high. Two normal sera and sera from two patients with Hodgkin's disease were studied. The sera were first fractionated by preparative zone electrophoresis; these fractions were subsequently subfractionated by anion-exchange cellulose chromatography. At least 16 subfractions were obtained for each serum, and the serum level and hexose content were determined for each.

The elevation in total protein-bound hexose in the Hodgkin's disease sera could be attributed chiefly to three alpha globulin subfractions. One of these, an alpha$_2$ globulin, made the largest contribution to the hexose increase and also caused the increase in alpha$_2$ globulin seen on paper electrophoresis of the whole serum. Another contained the alpha$_1$ acid glycoprotein, orosomucoid. The third was an alpha$_1$ globulin but was not characterized beyond its chromatographic behavior and hexose content. The beta and gamma globulins accounted for the remainder of the increase in protein hexose in the abnormal sera but no distinctive pattern of change was found in the subfractions except for a decrease in the siderophilin containing beta globulin component in the abnormal sera. There was also a decrease of the albumin and an increase of the ceruloplasmin containing alpha$_2$ globulin component in the sera from the patients with Hodgkin's disease.

AN INCREASE in protein-bound carbohydrate is one of the more striking changes that may occur in the serum protein as a reflection of disease. Electrophoresis studies have shown that most of the increase in serum protein-bound carbohydrate in a variety of neoplastic and inflammatory conditions is in the alpha globulins.[1-7] However, it is known that each of the globulins separated by usual electrophoretic methods contains more than one protein[8-12] and the changes in more highly resolved components within the electrophoretic fractions remain largely unexplored. It is hoped that delineation of the specific proteins involved will make clearer the significance of changes in serum protein-bound carbohydrate in disease.

In this study the distribution of carbohydrate among components within the electrophoretic fractions was investigated in a neoplastic disease. Hodgkin's disease was chosen for study because of the frequent association with high

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levels of serum protein-bound carbohydrate.[13, 14] Hexose was selected as a representative carbohydrate because it constitutes the largest portion of the carbohydrate in the serum protein.[15] Subfractionation of electrophoretically separated globulins was carried out by cellulose anion-exchange chromatography and the results compared for normal sera and sera from patients with Hodgkin's disease.

MATERIALS AND METHODS

Specimen Source.—Four individual sera were studied; one each from a normal female (I) and male subject (II), and from a female (III) and male (IV) patient with Hodgkin's disease. The diagnosis of Hodgkin's disease was made by lymph node biopsy. The two patients with this disease had no evidence of infection or other complication and had not received treatment for their disease. All sera were obtained during the fasting state of the subject and were kept frozen until used.

Initial Fractionation—Electrophoresis.—Fractions were prepared by zone electrophoresis in blocks of polyvinyl chloride powder* using a procedure modified from Müller-Eberhard and Kunkel.[16] Block sizes varied from 6.5 by 20 by 0.5 cm. to 20 by 20 by 0.7 cm. Electrophoresis was carried out in barbital buffer 0.075 μ, pH 8.6, with a constant current fixed between 20 and 50 Ma. The serum was first dialyzed against the buffer to remove free sugar. One to 5 ml. serum was applied to a single block, the amount depending upon the size of the block.

The protein was divided into five fractions: albumin, alpha$_1$, alpha$_2$, beta and gamma globulins. In some cases the division was on the basis of the peaks in relative protein concentration as measured by the absorption at 286 mμ of the eluate from serial segments of the block. This wavelength was used rather than the usual 280 mμ in order to minimize the absorption caused by barbital buffer. The remaining blocks were divided directly into five areas determined by the pigment bands. The albumin area could be identified by a yellow color. The blue band of ceruloplasmin was in the leading portion of the alpha$_2$ globulin zone. If hemolysis had occurred the reddish brown color of the hemoglobin-haptoglobin complex appeared between the alpha$_2$ and beta globulin zones at the trailing boundary of the alpha$_2$ globulin. The pink band of siderophilin was behind this and marked the first portion of the beta globulin; it was followed closely by an orange color, also in the beta globulin zone. No pigment could be seen in the alpha$_1$ and gamma globulin zones.

To obtain sufficient quantities of the electrophoretic fractions each serum was separated on several blocks and the corresponding fractions were pooled. The total amounts fractionated electrophoretically were: 12 ml. serum I, 9.6 ml. serum II, 12 ml. serum III, and 20 ml. serum IV. Aliquots from these pools were removed for analysis and the remainder was chromatographed.

Subfractionation—Chromatography.—Each of the electrophoretic fraction pools was subfractionated by chromatography using the anion exchanger, diethylaminoethyl (DEAE) cellulose, of Peterson, Sober, and co-workers.[11, 17] The modification of their chromatographic method using 3 Gm. of the adsorbent has been presented in detail elsewhere.[18] A small amount of "euglobulin" which precipitated during dialysis of the electrophoretic gamma globulin fractions against the starting buffer (0.01M phosphate, pH 8.0) was removed and analyzed for protein and hexose. Since a large portion of the gamma globulins did not bind to the cellulose at the starting conditions and would otherwise have emerged in a large volume of effluent, the gamma globulin fraction was concentrated to 2 to 4 ml. by ultrafiltration[11] prior to application to the column. All other fractions were completely adsorbed under the starting conditions and were permitted to concentrate on the column. The proteins were eluted by a continuous gradient system of increasing phosphate molarity and decreasing

*Geon resin type 426, obtained from the B. F. Goodrich Chemical Co., Avon Lake, Ohio.

TABLE I. SERUM CONCENTRATION AND HEXOSE CONTENT OF COMPONENTS IN SERUM FROM NORMAL SUBJECTS SEPARATED BY ELECTROPHORESIS AND CHROMATOGRAPHY

	ZONE													
	A		B		C		D		E		F		TOTAL	
FRACTION	PROTEIN CONCENTRATION (MG./ML.)	H/P	PROTEIN CONCENTRATION (MG./ML.)	H/P	PROTEIN CONCENTRATION (MG./ML.)	H/P	PROTEIN CONCENTRATION (MG./ML.)	H/P	PROTEIN CONCENTRATION (MG./ML.)	H/P	PROTEIN CONCENTRATION (MG./ML.)	H/P	PROTEIN (MG./ML.)	TOTAL HEXOSE (MG./ML.)
Serum I (female)														
Albumin							51.	0.0002					51.	0.01
α_1									1.7	0.059	1.3	0.13	3.0	0.27
$\alpha_{1,2}$									0.2	0.1			0.2	0.02
α_2							2.6	0.060	1.9	0.063	0.50	0.039	5.0	0.30
α_3									1.4	0.03			1.4	0.04
β					4.3	0.030	0.6	0.05	0.9	0.03			5.8	0.19
γ	10.0	0.011	0.6	0.016	1.4	0.012	0.3	0.017	0.9	0.038			13.	0.18
										Precipitate* (H/P = 0.03)			= 0.26	0.08
										Total			80.	1.09
Serum II (male)														
Albumin							57.	0.0002					57.	0.01
α_1									1.0	0.06	1.4	0.12	2.4	0.23
$\alpha_{1,2}$									0.6	0.1			0.6	0.06
α_2							2.5	0.054	1.9	0.048	1.3	0.04	5.7	0.28
α_3									0.6	-			0.6	-
β					3.2	0.029	0.6	0.05	1.2	0.03	1.0	0.03	6.0	0.19
γ	7.3	0.01	0.9	-	1.5	0.01	1.2	-	0.3	-			11.	0.09†
												Precipitate*	= 0.15	-
												Total	84.	0.86†

*Precipitate which formed in the electrophoretic gamma globulin fraction during preparation for chromatography (see Methods).
†Complete quantification was not possible.

pH,[18] beginning with sodium phosphate buffer, 0.01M, pH 8.0, and approaching a limit of 0.30M mono-sodium phosphate. The elution buffer was forced through the column with a peristaltic-type pump at a flow rate of 10 ml. per hour. The effluent was collected in 3 ml. fractions by a time actuated collector. The hexose concentration was determined for each of these fractions. The optical density at 280 mμ was used as an index of relative concentration of protein. The chromatographic fractions were pooled according to component boundaries suggested by the chromatograms. The total protein and hexose of each pool were then determined and each pool was examined by analytic electrophoresis.

Analytic.—A modification of the thymol–sulfuric acid procedure of Shetlar and Masters[19] was used for the determinations of protein-bound hexose. The sulfuric acid and thymol solutions were mixed 2 to 4 hours prior to use, thereby simplifying the procedure when a large number of determinations were made. The final proportions of the reagents, the conditions of the reaction, and the results were essentially the same as described in the published method. Corrections for errors from color caused by interaction between protein and acid and between hexose and acid were made by a system of blanks similar to that used in the carbazole method of Seibert and Atno.[20] A solution of equal proportions of mannose and galactose wase used as a standard. The reaction does not detect glucosamine. The mean level of normal human serum protein-bound hexose by this method, when determined on the ethanol precipitate of serum, was found to be 126 mg. per cent; 1 standard deviation equals 14 mg. per cent. The chromatographic effluent could be assayed directly since the adsorbent was found to contribute little or no detectable carbohydrate. When present this was corrected for by subtraction of the amount found in the effluent of the chromatogram prior to the application of the protein to the column.

Serum, electrophoretic fractions, and chromatogram pools were analyzed by zone filter paper electrophoresis in barbital buffer at 0.075 μ, pH 8.6.[18] Dilute mixtures were first concentrated by ultrafiltration.[11] Biuret methods were used for all determinations of total protein.[21, 22] The protein standard consisted of a solution of crystalline bovine albumin which had been assayed for nitrogen by the Kjeldahl method.

Calculations.—The hexose content for each component was expressed as the weight ratio of hexose to protein (H/P) as determined by the thymol–sulfuric acid and biuret reactions. This could be calculated directly for single component pools. For pools which proved to be mixtures by analytic paper electrophoresis the calculations employed the assumption that components which appeared at the same point in the effluent of corresponding chromatograms and which had the same electrophoretic mobility were identical. With this assumption the hexose to protein ratios for the remaining components could be calculated.

RESULTS

The total protein-bound hexose was markedly elevated in the sera from the two patients with Hodgkin's disease. The values obtained on the ethanol precipitate of whole serum were 300 mg. per cent and 302 mg. per cent for Hodgkin's disease sera III and IV, respectively. The corresponding values of 132 mg. per cent and 121 mg. per cent for normal sera I and II were well within the normal range. The preliminary fractionation by electrophoresis showed that most of the increase in hexose was within the alpha globulins (Tables I and II, final column) as has been found by others.[1-7]

The chromatograms for the electrophoretic fractions of the serum from normal subject I and from patient IV with Hodgkin's disease are given in Fig. 1. Curves for 2 ml. of the whole sera obtained by the same chromatographic procedure are shown at the top for comparison. The resemblance of the whole serum chromatogram to a summation of the corresponding component chromatograms below it is apparent. The chromatograms for the remaining sera are qualitatively similar and are not illustrated.

TABLE II. SERUM CONCENTRATION AND HEXOSE CONTENT OF COMPONENTS IN SERUM FROM PATIENTS WITH HODGKIN'S DISEASE SEPARATED BY ELECTROPHORESIS AND CHROMATOGRAPHY

	ZONE													
	A		B		C		D		E		F		TOTAL	
FRACTION	PROTEIN CONCENTRATION (MG./ML.)	H/P	PROTEIN CONCENTRATION (MG./ML.)	H/P	PROTEIN CONCENTRATION (MG./ML.)	H/P	PROTEIN CONCENTRATION (MG./ML.)	H/P	PROTEIN CONCENTRATION (MG./ML.)	H/P	PROTEIN CONCENTRATION (MG./ML.)	H/P	TOTAL PROTEIN (MG./ML.)	TOTAL HEXOSE (MG./ML.)
Serum III (female)														
Albumin							34.	0.002					34.	0.07
α_1									3.4	0.080	2.3	0.20	5.7	0.73
$\alpha_{1,2}$									1.	0.09			1.	0.09
α_2							3.8	0.040	6.1	0.086	1.5	0.049	11.	0.75
α_3									1.5	0.05			1.5	0.08
β					2.0	0.045	4.6	0.04			2.5	0.03	9.1	0.35
γ	9.0	0.011	1.6	0.014	3.1	0.014	1.1	0.019	1.4	0.04			16.	0.24
										Precipitate (H/P = 0.04) =			1.5	0.06
										Total			80.	2.37
Serum IV (male)														
Albumin							32.	0.0003					32.	0.01
α_1									3.6	0.057	2.6	0.20	6.2	0.73
$\alpha_{1,2}$									0.1	–			0.1	–
α_2							2.8	0.041	9.4	0.073	2.4	0.057	15.	0.94
α_3									2.9	0.02			2.9	0.06
β					1.2	0.022								
					1.1	0.066	2.8	0.04			2.9	0.03	8.0	0.30
γ	9.0	0.011	1.6	0.013	2.1	0.014	0.9	0.025	1.4	0.032			15.	0.22
										Precipitate (H/P = 0.02) =			0.5	0.01
										Total			80.	2.27*

Two separate components appeared in zone C of the β fraction of serum IV. These can be seen as the two peaks in the chromatogram illustrated in Fig. 1.

*Complete quantification was not possible.

Because of the overlapping of zones in the preliminary electrophoretic fractionation several of the fractions were electrophoretically heterogeneous. for example, the small amount of protein in zone D of the alpha$_1$ globulin curve for the normal serum illustrated in Fig. 1 proved to be an alpha$_2$ globulin. Similarly, the component which appeared in zone C of the alpha$_2$ chromatogram of this serum was a beta globulin. With the data from analytic electrophoresis of the chromatogram pools corrections could be made for these contaminants.

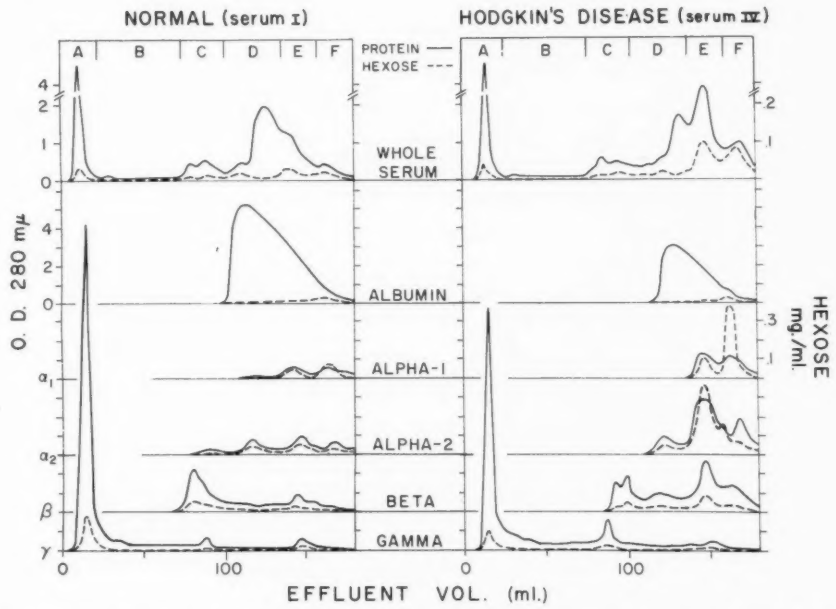

Fig. 1.—The chromatograms of serum and serum electrophoretic fractions from a normal subject (serum I), and from a patient with Hodgkin's disease (serum IV) are illustrated. The chromatograms for 2 ml. of the whole sera are at the top. Below them are the curves for the five electrophoretic fractions from 12 ml. of the same sera. The relative protein concentration (O.D. 280mμ) of the effluent is indicated by the solid line; the hexose concentration by the broken line. (The ordinate scale for the whole serum is one half that for the fractions.) Arbitrary zone divisions of the chromatograms are indicated at the top by the letters *A* to *F*. Zone boundaries are at 27, 78, 105, 140, and 162 ml. effluent volume.

The calculated serum level for each component with its hexose content is given in Tables I and II. The data are arranged according to electrophoretic mobility and the chromatographic zone in which the greater part appeared. The total serum protein hexose calculated from the chromatogram fraction measurements (final column, Tables I and II) appeared to be about 80 per cent of the value obtained directly on an ethanol precipitate of the same serum. It is not known, however, whether fraction analyses were too low or whether the whole serum values were high.

All of the electrophoretic globulin groups were subdivided into two or more components. These components have been noted in previous studies of normal human sera.[11, 18] In presenting the results of this study each component has been characterized by the hexose to protein ratio and by the individual proteins known to be present in it.

Albumin.—Albumin appeared principally as a single component but continued to emerge to the end of the chromatogram as has been found previously with DEAE cellulose chromatograms.[11, 18] Hexose was absent in the greater portion of the albumin. A small amount of hexose appeared in the latter part of the chromatograms (Fig. 1) and was due, at least in part, to contamination of the original electrophoretic albumin fractions with trace amounts of $alpha_1$ globulins. The total amount of albumin was decreased in the Hodgkin's disease sera tested.

$Alpha_1$ Globulins.—Two distinct chromatographic components were obtained from this electrophoretic group (Fig. 1). The first of these, in zone E, had a hexose to protein ratio of approximately 0.06 except for one of the abnormal sera, in which it was slightly higher. This component probably contains the $alpha_1$ lipoprotein.[23] The abnormal sera contained 2 to 3 times as much of this component as the normal sera.

The second chromatographic component, zone F, contains the acid glycoprotein or orosomucoid.[11, 24, 25] The normal hexose to protein ratios of 0.12 and 0.13 for the $alpha_1$ zone F are lower than the reported values of 0.16 and 0.18 for purified orosomucoid which suggests that other $alpha_1$ globulins may be present in this chromatographic component. In the Hodgkin's disease sera the protein of the $alpha_1$ zone F was increased and, in addition, the hexose content of this fraction was increased. The rise to a hexose to protein ratio of 0.20 in Hodgkin's disease is compatible with a relative increase of carbohydrate-rich proteins, e.g., orosomucoid, in this fraction.

$Alpha_{1,2}$ Globulin.—A component of electrophoretic mobility between that of the $alpha_1$ and $alpha_2$ globulins, designated $alpha_{1,2}$ globulin, appeared regularly in the latter part of zone E. Most of it was included within the $alpha_2$ fractions in the preliminary electrophoretic fractionation of serum. It can be detected in the $alpha_2$ globulin chromatograms of Fig. 1 as a small peak in protein and hexose appearing on the descending portion of the second peak. The hexose content of 0.09 to 0.1 places this component second only to the $alpha_1$ zone F component in hexose content. The small amount and the difficulties in separation of this component prevented reliable quantitative appraisal of the possible changes in Hodgkin's disease although its peak appeared more prominent in the chromatograms of the abnormal sera.

$Alpha_2$ Globulins.—Three $alpha_2$ globulin components could be distinguished. The first component, $alpha_2$ zone D, was observed to contain hemoglobin if slight hemolysis had occurred during collection of the blood, indicating that the haptoglobins, $alpha_2$ hemoglobin binding proteins,[9, 11, 26] were associated with this component. The normal hexose to protein ratios in $alpha_2$ zone D were 0.060 and 0.054. In the Hodgkin's disease sera the amount of protein in this component appeared to be somewhat higher than in the normal subjects whereas the hexose to protein ratios were lower.

Although little is yet known about the $alpha_2$ zone E component it exhibited the most marked differences from the normal in this study. There was 3 and

5 times the normal amount of this component in the Hodgkin's disease sera III and IV, respectively. In addition, the hexose to protein ratios were higher than in the normal subjects. This component was responsible for the major abnormality in the Hodgkin's disease whole serum chromatograms as seen in the high peaks in protein and hexose in zone E for serum IV at the top of Fig. 1.

The third alpha$_2$ globulin component, zone F, was blue in color and contained ceruloplasmin.[11] The hexose to protein ratios of 0.039 and 0.040 are in agreement with the value of 0.04 obtained for purified ceruloplasmin.[10, 27] With the sera from patients with Hodgkin's disease, the blue band on the column during chromatography was noted to be greater than in the normal sera as was also the quantity of protein in the alpha$_2$ zone F component.

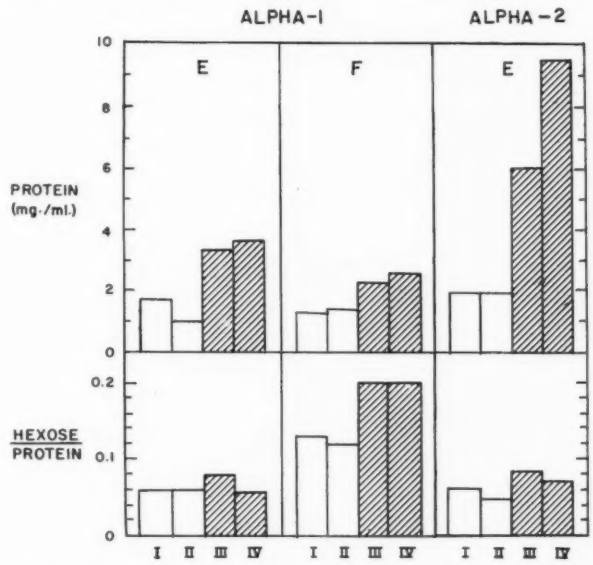

Fig. 2.—Comparison of the alpha globulin components, showing the greatest changes in Hodgkin's disease. The serum protein level for each component is given in the upper columns, as milligram protein per milliliter serum. The lower columns represent the hexose to protein ratios. The letter at the top of each group refers to the chromatogram zone (see Fig. 1). Open columns are used for the normal sera and shaded columns for the Hodgkin's disease sera.

Alpha$_3$ Globulin.—A component with electrophoretic mobility between that of the alpha$_2$ and beta globulins appeared in zone E of the alpha$_2$ and beta chromatograms and is referred to as alpha$_3$ globulin. In Fig. 1 it is the principal component of the prominent peak in zone E of the beta chromatogram of serum IV. The hexose to protein ratio of this component was in the order of 0.02 to 0.05. Whether or not the concentration of this protein was altered in Hodgkin's disease was not determinable.

Beta Globulins.—This group exhibited the greatest variation among the four sera. At least three beta globulin components could be distinguished for each serum. The first component contained the siderophilin or transferrin.[11, 18] The level of this zone C component was lower in the two abnormal sera. In only one serum (IV) did the hexose content of this component approach the value of 0.018 reported for purified siderophilin.[28]

The results suggest that a small but significant portion of the increase in serum protein hexose in the Hodgkin's disease sera came from the beta globulins (final column, Tables I and II). Because of poor resolution of some of the components and variation among the normal and abnormal sera, however, no clear pattern of change in Hodgkin's disease was evident.

Gamma Globulins.—This group of proteins was uniformly divided into five fractions. The ultracentrifugal and immunochemical characteristics of these fractions have been investigated.[29] A progressive increase in the hexose content in the consecutive fractions has also been demonstrated[29] and this is seen, with few exceptions, in the present study. The relatively high hexose to protein ratio for the zone E fraction is attributed to the 18S gamma macroglobulin, which is contained therein.[12, 16, 29]

The quantity of protein in fractions B, C, and E, and in the total gamma globulin was higher in the two patients with Hodgkin's disease than in the normal subjects. However, the protein concentrations in the fractions were so low that precise measurements were not possible and these differences can be regarded only as tentative. The hexose to protein ratios of the gamma globulin fractions did not appear to be altered in Hodgkin's disease.

DISCUSSION

The total serum protein-bound carbohydrate level is a fairly nonspecific measure of the extent or activity of many diseases with an inflammatory response or neoplastic growth.[30] Several studies have indicated that measurement of total protein-bound carbohydrate or certain serum fractions of high carbohydrate content may be of somewhat greater clinical value than determination of erythrocyte sedimentation rate or C-reactive protein in disease.[14, 31-33] Tests for protein-bound carbohydrate may, however, be more useful clinically when variations in single serum proteins can be measured.

Electrophoresis studies have demonstrated that most of the increase in protein-bound carbohydrate is in the alpha globulins.[1-7] In the present study three alpha globulin subfractions exhibited the greatest differences between the normal and abnormal sera. The data for the three are summarized for comparison in Fig. 2. These three alpha globulin components together accounted for the major portion of the increase in serum protein-bound hexose in the Hodgkin's disease sera. The $alpha_2$ zone E component was principally responsible for the increase in $alpha_2$ globulin in the Hodgkin's disease sera and also made the greatest single contribution to the increase in total serum protein-bound hexose. The $alpha_1$ zone F contained orosomucoid, which has been shown by immunochemical methods to be elevated in disease.[34, 35] The $alpha_1$ zone E remains incompletely characterized but its hexose to protein ratio is similar to that reported for several $alpha_1$ globulin subfractions by Schultze and coworkers.[10]

Some of these differences between the alpha globulins in the disease and normal sera have been confirmed by protein analyses on six additional normal sera and two additional Hodgkin's disease sera using initial chromatography

with subfractionation by electrophoresis.[23] However, further studies will be required before it is known whether this pattern of change is unique to Hodgkin's disease or whether it is as nonspecific an accompaniment of disease as the elevation of the total serum protein-bound hexose.

The beta and gamma globulins accounted for the remainder of the increase in protein hexose in the abnormal sera with somewhat more from the beta globulins. However, a characteristic pattern of change in the distribution of hexose among the subfractions of either of these groups was not found. In addition, several chromatographic components were altered in Hodgkin's disease but did not contribute significantly to the changes in total protein-bound hexose. The low albumin was not surprising since low levels are common in this as in other neoplastic diseases. The low siderophilin containing beta C component may reflect a decrease in the siderophilin itself, which would be consistent with the fall in serum total iron binding capacity commonly seen in neoplastic disease.[36] Ceruloplasmin, on the other hand, appeared to be increased in the Hodgkin's disease sera. This was suggested by the finding of increased protein in the ceruloplasmin containing $alpha_2$ zone F component and an increased intensity of the blue band noted on the columns. Elevated serum copper has been reported with this disease.[37]

The physicochemical properties of most of these components have not as yet been studied. As such information becomes available the relation of these to subfractions prepared by other methods will become clearer. Also, the purity of the subfractions must be further defined. Some of the components may prove to be single protein moieties and others may be found to be mixtures of proteins with electrophoretic and chromatographic properties in common. Inhomogeneity of some components has been implied earlier in this article in relation to observed differences in hexose to protein ratio for the same component from different sera (e.g., $alpha_1$ zone F). Differences in hexose to protein ratio could also be due to actual qualitative differences in homogeneous proteins or to limitations in the precision of the analytic techniques.

The marked changes that may occur in serum protein components in Hodgkin's disease are emphasized by the present study. Disease complications that might have been responsible for serum protein alterations were avoided by using sera from patients who had received no treatment and who had no detectable infection or liver disease. How Hodgkin's disease effects the changes seen in the serum proteins is largely unknown and there is scanty information concerning the normal metabolism of the carbohydrate-rich proteins. Suggestions have been made that the latter originate in tumor sites[38] or in the liver[39] but the evidence is incomplete. Recent experiments suggest that there is a rapid turnover in the carbohydrate portion of orosomucoid[40] but the function of this as well as of other glycoproteins remains to be clarified.

SUMMARY

The changes in serum proteins and protein-bound hexose were investigated by electrophoresis and anion-exchange cellulose chromatography in two patients

with Hodgkin's disease. Sera from two normal subjects were used for comparison. The most marked differences were in three alpha globulin components, which were also responsible for most of the increase in total protein-bound hexose in the Hodgkin's disease sera. One $alpha_2$ globulin component contributed the largest amount of hexose and also caused the elevation in total $alpha_2$ globulin detected by ordinary electrophoresis of the serum. An $alpha_1$ globulin component which contained orosomucoid made the second largest contribution to the hexose increase in the abnormal sera. In addition, the ceruloplasmin containing component was increased in the sera from the patients with Hodgkin's disease whereas the albumin and siderophilin containing components were decreased.

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REFERENCES

1. Seibert, F. B., Seibert, M. V., Atno, A. J., and Campbell, H. W.: Variation in Protein and Polysaccharide Content of Sera in the Chronic Diseases, Tuberculosis, Sarcoidosis and Carcinoma, J. Clin. Invest. **26**: 90, 1947.
2. Wunderly, C., and Piller, S.: The Staining of Serum Proteins, Lipids, and Carbohydrates After Electrophoresis, Klin. Wchnschr. **32**: 425, 1954.
3. Goa, J.: On Protein-bound Carbohydrates in Human Serum; a Study on Hexosamine and Hexose Content of Electrophoretically Separated Fractions of Normal and Pathological Sera, Scandinav. J. Clin. & Lab. Invest. **7**, suppl. 22: 1, 1955.
4. Roboz, E., Hesse, W. C., and Forster, F. M.: Determination of Serum Proteins and Polysaccharides; Comparison of Neoplastic Diseases, Particularly of the Central Nervous System, A.M.A. Arch. Neurol. & Psychiat **73**: 536, 1955.
5. Puls, W., and Fiedler, L.: The Polysaccharide Content of Serum Proteins in Health and Disease, Clin. chim. acta **1**: 348, 1956.
6. Stidworthy, G., Payne, R. W., Shetlar, C. L., and Shetlar, M. R.: Objective Evaluation of Patients With Rheumatic Diseases. II. Paper Electrophoretic Studies of Serum Glycoprotein and Protein From Patients With Rheumatoid Arthritis, J. Clin. Invest. **36**: 309, 1957.
7. Almquist, P. O., and Lausing, E.: A Study of Serum Glycoproteins in Cancer, Scandinav. J. Clin. & Lab. Invest. **9**: 179, 1957.
8. Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, G., Gillespie, J. M., Kahnt, F. W., Lever, W. F., Liu, C. H., Mittelman, D., Mouton, R. F., Schmid, K., and Urona, E.: A System for the Separation of the Components of Human Blood: Quantitative Procedures for the Separation of the Protein Components of Human Plasma, J. Am. Chem. Soc. **72**: 465, 1950.
9. Smithies, O.: Zone Electrophoresis in Starch Gels: Group Variation in the Serum Proteins of Normal Human Adults, Biochem. J. **61**: 629, 1955.
10. Schultze, H. E., Göllner, I., Heide, K., Schönenberger, M., and Schwick, G.: The Alpha Globulins of Normal Human Serum, Ztschr. Naturforsch. **106**: 463, 1955.
11. Sober, H. A., Gutter, F. J., Wyckoff, M. M., and Peterson, E. A.: Chromatography of Proteins. II. Fractionation of Serum Protein on Anion-Exchange Cellulose, J. Am. Chem. Soc. **78**: 756, 1956.
12. Wallenius, G., Trautman, R., Kunkel, H. G., and Franklin, E. C.: Ultracentrifugal Studies of Major Non-lipide Electrophoretic Components of Normal Human Serum, J. Biol. Chem. **225**: 253, 1957.
13. Lockey, E., Anderson, A. J., and Maclagan, N. F.: Urine and Serum Mucoproteins in Cancer and Other Diseases, Brit. J. Cancer **10**: 209, 1956.
14. Falholt, W., Menini, E., and Lous, P.: Seromucoid and Protein-bound Hexoses in Serum. II. Diagnostic Value and Comparison With Paper-Electrophoretic Fractionation of Serum-Proteins, Acta med. scandinav. **160**: 323, 1958.
15. Winzler, R. J.: Determination of Serum Glycoproteins, *in* Glick, D., editor: Methods of Biochemical Analysis, New York, 1955, Interscience Publishers, Inc., vol. 2, pp. 279-311.
16. Müller-Eberhard, H. J., and Kunkel, H. G.: The Carbohydrate of Gamma-Globulin and Myeloma Proteins, J. Exper. Med. **104**: 253, 1956.

17. Peterson, E. A., and Sober, H. A.: Chromatography of Proteins. I. Cellulose Ion-Exchange Adsorbents, J. Am. Chem. Soc. **78**: 751, 1956.
18. Fahey, J. L., McCoy, P. F., and Goulian, M.: Chromatography of Serum Proteins in Normal and Pathologic Sera: the Distribution of Protein-bound Carbohydrate and Cholesterol, Siderophilin, Thyroxin-Binding Protein, B_{12}-Binding Protein, Alkaline and Acid Phosphatases, Radioiodinated Albumin and Myeloma Proteins, J. Clin. Invest. **37**: 272, 1958.
19. Shetlar, M. R., and Masters, Y. F.: Use of the Thymol-Sulfuric Acid Reaction for Determination of Carbohydrates in Biological Material, Anal. Chem. **29**: 402, 1957.
20. Seibert, F. B., and Atno, J.: Determination of Polysaccharide in Serum, J. Biol. Chem. **163**: 511, 1946.
21. Mehl, J. W.: The Biuret Reaction in the Presence of Ethylene Glycol, J. Biol. Chem. **157**: 173, 1945.
22. Gornall, A. G., Bardawill, C. J., and David, M. M.: Determination of Serum Proteins by Means of the Biuret Reaction, J. Biol. Chem. **177**: 751, 1949.
23. Fahey, J. L.: To be published.
24. Weimer, H. E., Mehl, J. W., and Winzler, R. J.: Studies on the Mucoproteins of Human Plasma. V. Isolation and Characterization of a Homogeneous Mucoprotein, J. Biol. Chem. **185**: 561, 1950.
25. Schmid, K.: Preparation and Properties of Serum and Plasma Proteins. XXIX. Separation From Human Plasma of Polysaccharides, Peptides and Proteins of low Molecular Weight. Crystallization of an Acid Glycoprotein, J. Am. Chem. Soc. **75**: 60, 1953.
26. Nyman, M.: Serum Haptoglobin; Methodological and Clinical Studies, Scandinav. J. Clin. & Lab. Invest. **11**, Suppl. 39: 1, 1959.
27. Kominz, D. R.: Personal communication.
28. Koechlin, B. A.: Preparation and Properties of Serum and Plasma Proteins. XXVIII. The beta-1 Metal-Combining Protein of Human Plasma, J. Am. Chem. Soc. **74**: 2649, 1952.
29. Fahey, J. L., and Horbett, A. P.: Human Gamma Globulin Fractionation on Anion Exchange Cellulose Columns, J. Biol. Chem. **234**: 2645, 1959.
30. Greenspan, E. M.: Clinical Significance of Serum Mucoproteins, *in* Dock, W., and Snapper, I., editors: Advances in Internal Medicine, Chicago, 1955, Year Book Publishers, Inc., vol. 7, pp. 101-123, 1955.
31. Shetlar, M. R., Bullock, J. A., Shetlar, C. L., and Payne, R. W.: Comparison of Serum C-Reactive Protein, Glycoprotein, and Seromucoid in Cancer, Arthritis, Tuberculosis and Pregnancy, Proc. Soc. Exper. Biol. & Med. **88**: 107, 1955.
32. Shetlar, M. R., Payne, R. W., Padron, J., Felton, F., and Ishmael, W. K.: Objective Evaluation of Patients With Rheumatic Disease. I. Comparison of Serum Glycoprotein, Cold Hemagglutination, C-Reactive Protein, and Other Tests With Clinical Evaluation, J. LAB. & CLIN. MED. **48**: 194, 1956.
33. Cooke, W. T., Fowler, D. I., Cox, E. V., Gaddie, R., and Meynell, M. J.: The Clinical Significance of Seromucoids in Regional Ileitis and Ulcerative Colitis, Gastroenterology **34**: 910, 1958.
34. Silberberg, S., Goodman, M., Kefalides, N. A., and Winzler, R. J.: Immunochemical Determination of Orosomucoid, Proc. Soc. Exper. Biol. & Med. **90**: 641, 1955.
35. Goodman, M., Ramsey, D. S., Simpson, W. L., and Brennan, M. J.: The use of Chicken Antiserum for the Rapid Determination of Plasma Protein Components. II. The Assay of Human Serum Orosomucoid, J. LAB. & CLIN. MED. **50**: 758, 1957.
36. Laurell, C. B.: Studies on the Transportation and Metabolism of Iron in the Body, Acta physiol. scandinav. **14** Suppl. 46: 1, 1947.
37. Gubler, C. J.: Copper Metabolism in Man, J. A. M. A. **161**: 530, 1956.
38. Winzler, R. J., and Burk, D.: Blood Proteose and Cancer, J. Nat. Cancer Inst. **4**: 417, 1944.
39. Werner, I.: On the Regeneration of Serum Poly-saccharides and Serum Proteins in Normal and Intoxicated Rabbits, Acta physiol. scandinav. **19**: 27, 1949.
40. Boström, H., Rodén, L., and Yamashima, I.: On the Metabolism of the Acid Glycoprotein in Plasma, J. Biol. Chem. **230**: 381, 1958.

COMPARISON OF PROPERDIN TITERS IN SERA OF NORMAL SUBJECTS AND PATIENTS WITH LEUKEMIA AND LYMPHOMA BY ZYMOSAN AND BACTERIOPHAGE ASSAYS

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With the use of the zymosan assay titers of properdin were measured in sera of 100 normal subjects, 40 patients with leukemia, 56 patients with lymphoma or sarcoma, and 12 patients with multiple myeloma. The mean properdin titer in normal serum by this assay was 4 units per milliliter. Lower than normal mean titers were found in sera of patients with leukemia, lymphoma, sarcoma, and multiple myeloma.

Titers of properdin were measured with the use of the bacteriophage assay in sera of 150 normal subjects, 81 patients with leukemia, 85 patients with lymphoma or sarcoma, and 22 patients with multiple myeloma. The mean properdin titer in normal serum by this assay was 9 PhN_{50} per milliliter. Lower than normal mean titers were found in sera of patients with chronic lymphocytic leukemia, active lymphoma, active sarcoma, and multiple myeloma. Conversely, normal or higher than normal mean titers were found in sera of patients with chronic myelogenous leukemia, acute leukemia, inactive lymphoma, and inactive sarcoma.

No correlation of serum properdin titers was demonstrated between the zymosan and bacteriophage assays in sera of 63 normal subjects, 44 patients with leukemia, 58 patients with lymphoma or sarcoma, and 12 patients with multiple myeloma.

It is suggested that the lack of correlation between the two assays may be related to substances interfering with the inactivation of C'_3, the formation of properdin-zymosan complex, or inactivation of T_2r+ strain of Escherichia coli bacteriophage. Alternate theories suggest that antibodies to zymosan or to phage may explain the lack of correlation.

PROPERDIN, described in 1954 as a naturally occurring serum euglobulin, is said to be capable of bactericidal, virus inactivating, and hemolytic activity.[1] The zymosan assay of properdin is based on the ability of properdin to combine with polysaccharides, such as zymosan, forming a properdin-zymosan (PZ) complex, which at 37° C. in the presence of Mg^{++} and complement is said to inactivate the third component of complement (C'_3).[2] The activity of the remaining C'_3 can be measured with the use of sensitized sheep red blood cells. The bacteriophage assay of serum properdin is based on the ability of properdin to inactivate the T_2r+ strain of *E. coli* bacteriophage.[3-5]

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The purpose of this article is to present and compare properdin titers measured by the zymosan and bacteriophage assays in sera of normal subjects and patients with leukemia, lymphoma, sarcoma, and multiple myeloma.

MATERIALS AND METHODS

Serum Preparation.—Ten milliliters of blood was drawn aseptically, allowed to clot at room temperature for 20 to 40 minutes, and placed at 2° C. for 2 to 4 hours. After centrifugation at 2,200 g for 6 to 8 minutes, the supernatant serum was aspirated and aliquots were stored at −64° C. for 1 to 3 weeks before testing.

The zymosan assay was that of Pillemer and associates.[2] One modification was made, i.e., RP and R_3 reagents were prepared from a serum pool of 25 normal donors. The same lots of RP, R_3, and zymosan (7B 152) were used during the entire study.

The bacteriophage assay was that of Barlow and co-workers.[3-5] Phage neutralizing activity is standardized in terms of 50 per cent bacteriophage neutralization units (PhN_{50}) per milliliter of serum. A known standard serum was included in every day's experiments for both assays.

RESULTS

A. *Normal Subjects (Fig. 1).*—Titers of properdin were measured in sera of 100 normal subjects using the zymosan assay and 150 normal subjects using the phage assay. Seventy per cent of the subjects were men and 30 per cent were women. The ages ranged from 20 to 50 years. No significant difference in titer was found with respect to age and sex. With the zymosan assay properdin titers ranged from <1 to 10 units per milliliter with a mean of 4 units per milliliter. With the phage assay properdin titers ranged from <2 to 32.4 PhN_{50} per milliliter with a mean of 9 PhN_{50} per milliliter. Titers showed no change for at least 6 months when serum was stored at −64° C.

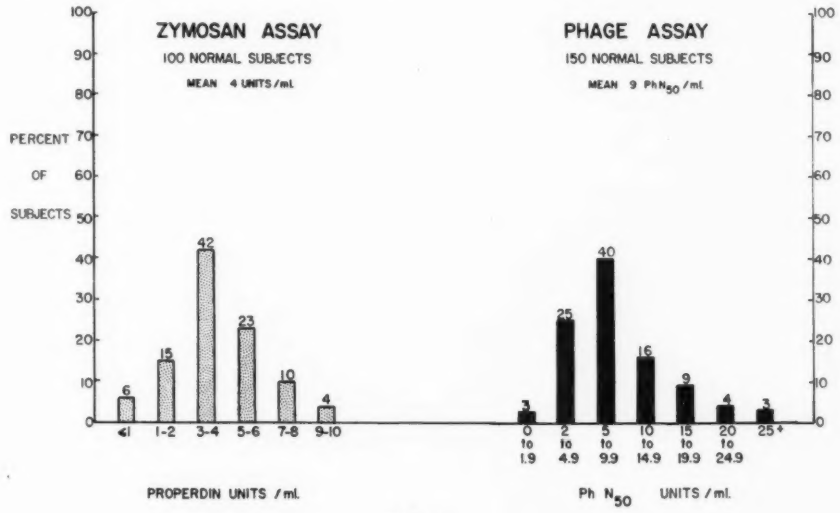

Fig. 1.

B. *Leukemia, Lymphoma, Sarcoma, and Multiple Myeloma.*—

Zymosan assay (Table I): Titers of properdin were measured in sera of forty patients with leukemia, of whom twenty-three had chronic lymphocytic,

ten had chronic myelogenous, and seven had acute leukemia. The mean values for all types of leukemia tested were low, i.e., 2.9 units per milliliter or less as compared with the normal of 4 units per milliliter. Serum properdin titers were measured in another group of sixty-eight patients, of whom thirty-three had Hodgkin's disease, twenty-three had lymphocytic or reticulum cell sarcoma, and twelve had multiple myeloma. These sixty-eight patients were divided into two categories, those with active and inactive disease. In the lymphoma and sarcoma group active disease was characterized by fever, anemia, adenopathy, and hepatomegaly with or without splenomegaly. In the myeloma group active disease was characterized by severe bone involvement on roentgenographic examination, pathologic fractures, high serum globulins, fever, or myeloma cells in the peripheral blood. Patients in the inactive phase of these diseases had none of these manifestations and had the diagnosis made by biopsy or bone marrow aspiration. The mean properdin values in both groups of patients were lower than normal. The lowest mean value was seen in patients with active multiple myeloma (<1 unit per milliliter). Similarly, mean properdin values in patients with active Hodgkin's disease were significantly low (1.6 units per milliliter). Conversely, a mean titer close to the normal value of 4 units per milliliter was found in sera of patients with inactive sarcoma (3.2 units per milliliter).

Phage assay (Table II): Titers of properdin were measured by this method in sera of eighty-one patients with leukemia, of whom forty-nine had chronic lymphocytic, twenty had myelogenous, and twelve had acute leukemia. The mean titer for patients with chronic lymphocytic leukemia was 2.8 PhN_{50} per milliliter as compared with the normal of 9 PhN_{50} per milliliter. Normal or higher than normal mean titers were seen in sera of patients with chronic myelogenous and acute leukemia (>10 PhN_{50} per milliliter). Properdin titers were measured in another group of one hundred and seven patients, of whom forty-six had Hodgkin's disease, thirty-nine had lymphocytic or reticulum cell sarcoma, and twenty-two had multiple myeloma. The mean titer for this group of patients with active disease was lower than normal. The lowest mean titers were seen in sera of patients with active Hodgkin's disease (3.5 PhN_{50} per milliliter) and active multiple myeloma (3.6 PhN_{50} per milliliter). Mean titers

TABLE I. ZYMOSAN ASSAY

DIAGNOSIS	NO. OF CASES		PROPERDIN (U./ML.) MEAN		RANGE		MEDIAN	
Normal	100		4		<1-10		4.4	
Leukemia								
Chronic lymphatic	23		2.9		<1-10		1.7	
Chronic myelogenous	10		1		<1-3		<1	
Acute	7		1.5		<1-6		<1	
Total	40							
	active	inactive	active	inactive	active	inactive	active	inactive
Hodgkin's disease	19	14	1.6	1.8	<1-5	<1-5	<1	1.2
Sarcoma	14	9	2.7	3.2	<1-10	<1-9	1.5	2.5
Lymphocytic								
Reticulum-cell								
Multiple myeloma	9	3	<1	1.6	<1-1	<1-5	<1	<1
Total	42	26						

TABLE II. PHAGE ASSAY

DIAGNOSIS	NO. OF CASES		pH N_{50} (U./ML.) MEAN		RANGE		MEDIAN	
Normal	150		9		<2-32		6.8	
Leukemia								
Chronic lymphatic	49		2.8		<2-7		1.8	
Chronic myelogenous	20		12.6		<2-28		8.7	
Acute	12		10.8		3-25		8.3	
Total	81							
	active	inactive	active	inactive	active	inactive	active	inactive
Hodgkin's disease	22	24	3.5	15.7	<2-19	3-48	3.4	12.5
Sarcoma	20	19	7	9.8	<2-28	3-40	4.3	9.0
Lymphocytic Reticulum-cell								
Multiple myeloma	12	10	3.6	6.1	<2-15	<2-14	1.3	6.5
Total	54	53						

approaching normal or higher than normal values were seen in patients with inactive Hodgkin's disease (15.7 PhN_{50} per milliliter) and inactive sarcoma (9.8 PhN_{50} per milliliter).

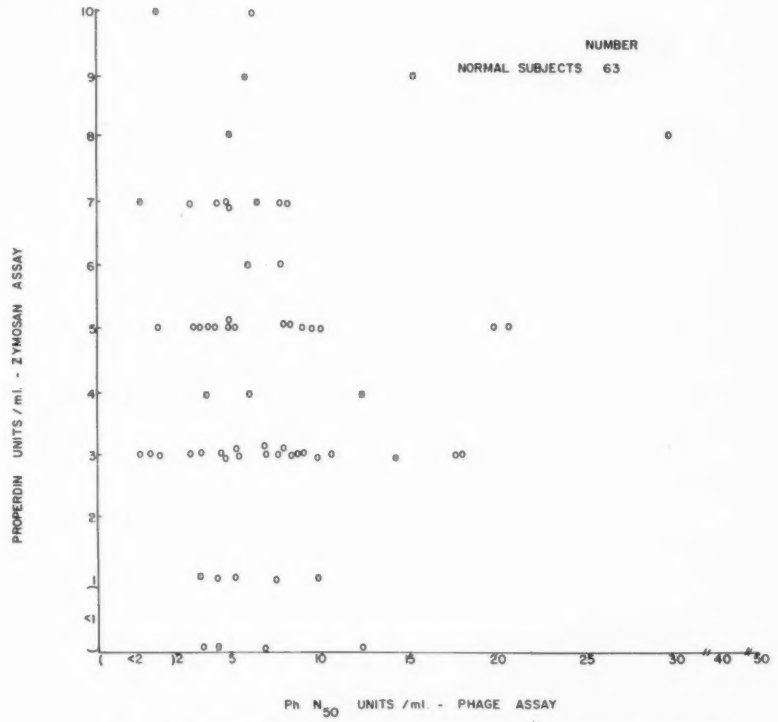

Fig. 2.—Comparison of zymosan and phage assays for properdin.

Figs. 2 to 5 show the lack of correlation between the zymosan and phage assays when aliquots of the same subject's serum were analyzed by both methods. In each of these figures the zymosan assay units are depicted on the ordinate and the phage units on the abscissa for the same subject's serum. The lack of correlation between the two assays was demonstrated both in the group of sixty-three normal subjects, and in the following groups of patients: forty-four

with leukemia, fifty-eight with Hodgkin's disease or sarcoma, and twelve with multiple myeloma. In addition, no correlation between the two assays could be demonstrated by a statistical calculation of the correlation coefficient (Table III).

TABLE III. CORRELATION COEFFICIENT OF ZYMOSAN AND PHAGE ASSAYS IN SERUM PROPERDIN DETERMINATIONS

DIAGNOSIS	NO. OF SERA	CORRELATION COEFFICIENT
Normal	63	0.126
Chronic lymphatic leukemia	23	0.123
Chronic myelogenous leukemia	8	0.53
Acute leukemia	9	0.15
Hodgkins, active	19	0.212
Hodgkins, inactive	15	0.474
Sarcoma, active	14	0.402
Sarcoma, inactive	10	0.363
Multiple myeloma, active	8	0.45
Multiple myeloma, inactive	3	0.5

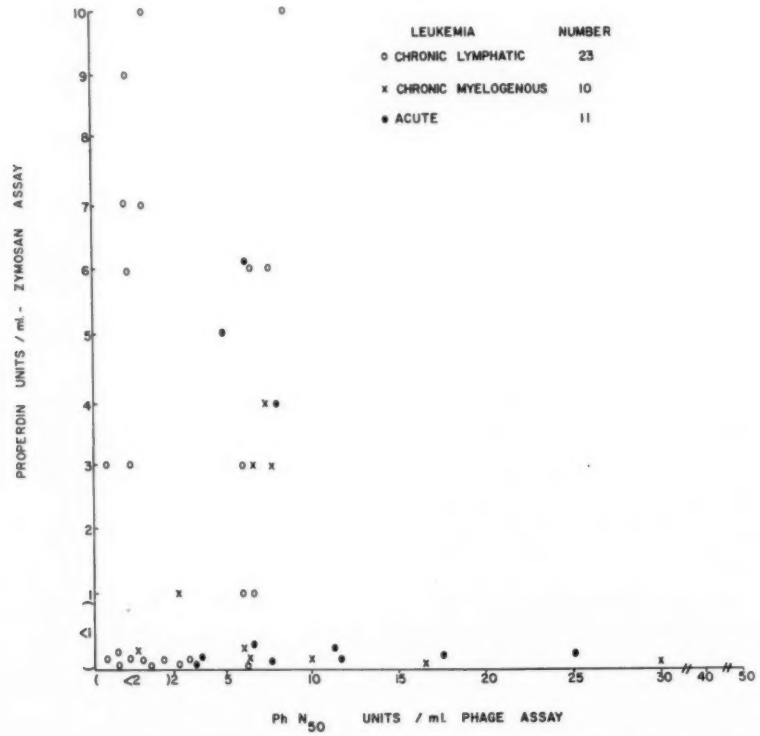
LEUKEMIA NUMBER
o CHRONIC LYMPHATIC 23
x CHRONIC MYELOGENOUS 10
• ACUTE 11
PROPERDIN UNITS / ml. - ZYMOSAN ASSAY
Ph N50 UNITS / ml. PHAGE ASSAY

Fig. 3.—Comparison of zymosan and phage assays for properdin.

DISCUSSION

From these data it is evident that no correlation could be found between the results of the two assays used for serum properdin determinations. Presuming that both assays are a measurement of the same substance, and that no interfering substances are present, one might expect the titers to be proportional

within the limit of error of both techniques. However, even when lower than normal mean titers are obtained by both assays, such as is true in sera of patients with chronic lymphocytic leukemia, the individual titer of any given serum is not comparable by both methods. A similar lack of correlation between the two assays in normal and a limited number of pathologic sera has been reported recently by Laurell and Reyn.[6, 7]

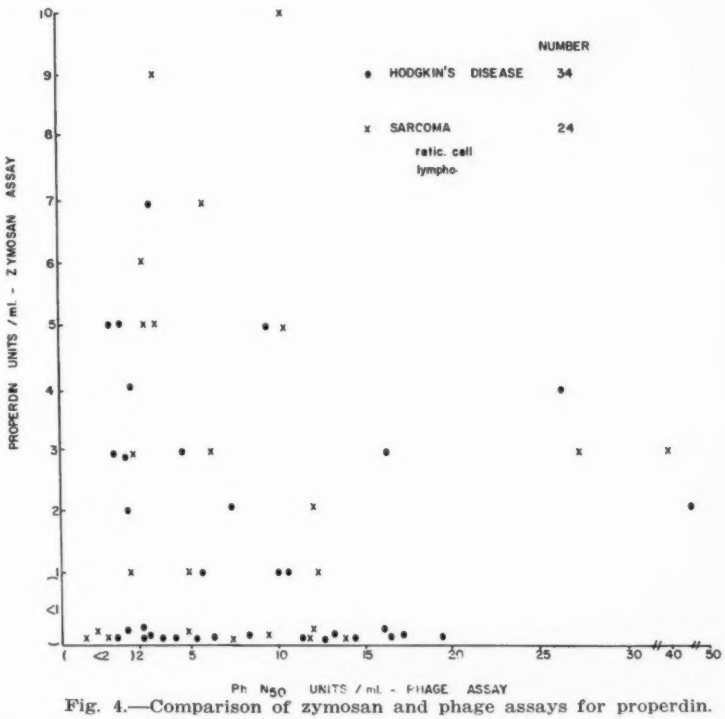

Fig. 4.—Comparison of zymosan and phage assays for properdin.

There are two possible explanations for the discrepancies between the results obtained by the zymosan and bacteriophage assays: (1) two different substances are measured; (2) the substance measured is the same, but additional factors may influence one or the other assay. The former hypothesis is thought to be unlikely because of the observation that properdin is necessary for the inactivation of T_2r^+ *E. coli bacteriophage*. Therefore, the lack of correlation between the two assays suggests the presence of substances which may influence one or the other assay. First, there is evidence that some human sera have factors which may interfere with the inactivation of C'_3[8] or the formation of properdin-zymosan complex.[9] These inhibitors could alter the results obtained by the zymosan assay. Similarly, there could be other serum factors which could interfere with the inactivation of T_2r^+ *E. coli* bacteriophage and alter the results obtained by the bacteriophage assay. Finally, there is evidence to suggest that properdin is an antibody to certain complex polysaccharides such as zymosan,[10] or that it may be a family of "specific" antibodies which are capable of reacting with certain bacteriophages.[11]

It seems possible that the two methods presently available for the measurement of serum properdin titers determine different parts of this complex globulin. If one assay measures one activity of this substance, a second assay determining another activity of the same substance could yield disparate results. The resultant differences need not necessarily suggest that an equal number of different substances were being detected by the two assays.

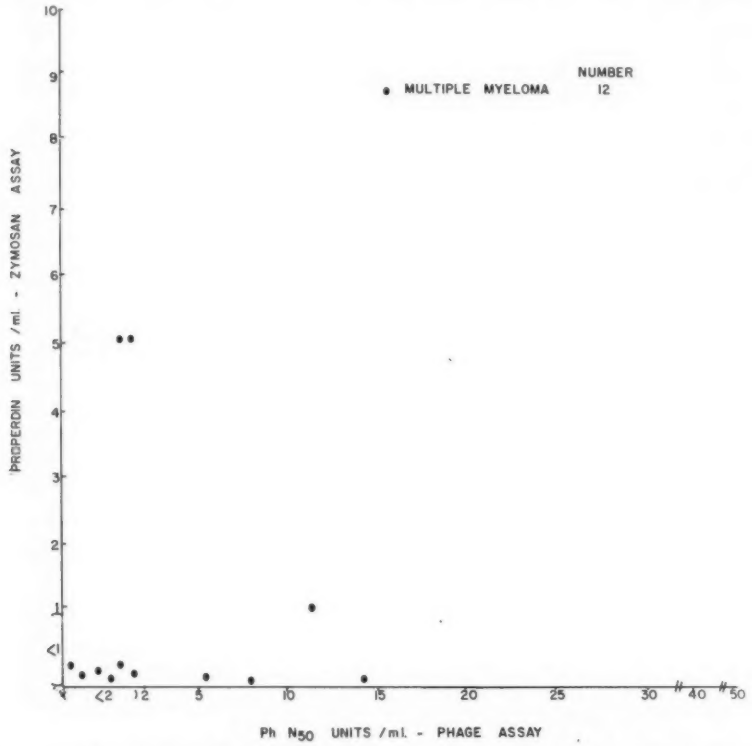

Fig. 5.—Comparison of zymosan and phage assays for properdin.

SUMMARY

Titers of properdin were measured in sera of normal subjects, patients with leukemia, lymphoma, sarcoma, and multiple myeloma using the zymosan and bacteriophage assays. The zymosan assay revealed low mean properdin titers in sera of patients with all types of leukemia, lymphoma, sarcoma, and multiple myeloma. Similarly, the phage assay revealed low mean properdin titers in patients with chronic lymphocytic leukemia, active lymphoma, sarcoma, and multiple myeloma. Conversely, normal or higher than normal mean titers with the phage assay were found in sera of patients with acute leukemia, chronic myelogenous leukemia, inactive lymphoma, and sarcoma.

No correlation of serum properdin titers was demonstrated between the zymosan and bacteriophage assays.

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REFERENCES

1. Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W., and Wardlaw, A. C.: Properdin System and Immunity. I. Demonstration and Isolation of a New Serum Protein, Properdin, and Its Role in Immunologic Phenomena, Science **120**: 279, 1954.
2. Pillemer, L., Blum, L., Lepow, I. H., and Warz, L.: The Properdin System and Immunity. III. The Zymosan Assay of Properdin, J. Exper. Med. **103**: 1, 1956.
3. Van Vunakis, H., Barlow, J. L., and Levine, L.: Neutralization of Bacteriophage by the Properdin System, Proc. Nat. Acad. Sc. **42**: 391, 1956.
4. Barlow, J. L., Van Vunakis, H., and Levine, L.: Studies on the Inactivation of Phage by the Properdin System. I. Evidence for Complement, Properdin and Magnesium Requirements, J. Immunol. **80**: 339, 1957.
5. Barlow, J. L., Van Vunakis, H., and Levine, L.: Studies on the Inactivation of Phage by the Properdin System. II. Quantitative Assay of Phage Neutralizing Activity, J. Immunol. **80**: 349, 1957.
6. Laurell, A. B., and Reyn, H.: Comparison Between Properdin Titers Obtained in the Zymosan Assay and in the Bacteriophage-Neutralization Assay. I. Investigation on Normal Human Sera, Acta path. et microbiol. scandinav. **47**: 405, 1959.
7. Laurell, A. B., and Reyn, A.: Comparison Between Properdin Titers Obtained in the Zymosan Assay and in the Bacteriophage-Neutralization Assay, Acta path. et microbiol. scandinav. **47**: 419, 1959.
8. Laurell, A. B.: Comparison of Two Serological Assays of Properdin in Normal Sera and Sera From Patients With Rheumatoid Arthritis and Cancer, Acta path. et microbiol. scandinav. **45**: 186, 1959.
9. Blum, L., and Lepow, I. H.: Demonstration of a New Factor in the Properdin System, Fed. Proc. **18**: 559, 1959.
10. Nelson, R. A.: An Alternative Mechanism for the Properdin System, J. Exper. Med. **108**: 515, 1958.
11. Cowan, K.: Interpretation of Properdin Levels Determined by Phage Neutralization Technic, Science **128**: 778, 1958.

IODINE METABOLISM IN THE THYROID

Influence of Thyrotropic Hormone, 6-Propylthiouracil, and Sodium Perchlorate

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A mince preparation of beef thyroid tissue when incubated in the presence of I^{131} has shown the ability to concentrate iodine, to increase iodine uptake in the presence of added thyroid stimulating hormone, and to form iodinated amino acids. When propylthiouracil was incorporated in the incubation media iodine uptake was diminished although an ability to respond to thyroid stimulating hormone remained. At greater concentrations of propylthiouracil, the formation of monoiodotyrosine and its subsequent iodination was markedly inhibited. In contrast sodium perchlorate, although resulting in a diminution in the iodine uptake of the mince preparation, had no effect on the distribution of thyroidal I^{131}. These data demonstrate that propylthiouracil has a direct effect not only on the uptake of radioiodine but also on the subsequent synthesis of iodotyrosines.

MANY investigators have studied the effect of 6-propylthiouracil and sodium perchlorate on the metabolism of thyroid tissue. These agents both have a depressing effect on thyroidal uptake of I^{131}. However their mechanism of action appeared to be different. Perchlorate has been shown to decrease total iodine concentration without influencing the formation of iodinated amino acids.[1, 2] Anbar[3] has suggested that perchlorate and iodide ions are the same volume and charge and therefore compete for the enzymes associated with iodine uptake. On the other hand propylthiouracil induces defective synthesis of iodotyrosines and thyronines. Richards and Ingbar[2] and Slingerland and associates[4] demonstrated that propylthiouracil decreased iodine uptake as well as the synthesis of radioactive amino acids. In the rat thyroid this agent limited the iodination of monoiodotyrosine since in its presence there was an increase in the ratio of monoiodotyrosine to diiodotyrosine. Because of such in vivo responses to this antithyroid compound it was of interest to study its effect on thyroid tissue in vitro. Under the latter conditions concentration of the inhibitor and thyrotropic hormone can be controlled and the effect of each evaluated in the absence of the other. This also permitted determination of the specific effect of the inhibitor and its relation to thyroid stimulating hormone.

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MATERIALS AND METHODS

Minces were prepared from beef thyroid and incubated with 20 μc I^{131} in a manner previously described.[5] The media was supplemented with 6-propylthiouracil or sodium perchlorate at 10^{-3} to 10^{-7} M. Radioiodine uptake and utilization were measured in both the absence and presence of thyrotropin (20 mu. per milliliter). After the radioactive minces were washed three times with buffer, uptake of I^{131} was determined using a probe-type scintillation counter at a fixed distance from the tissue. Iodine uptake was expressed as per cent of radioactivity added to the incubation medium.

The mince was prepared for chromatography by homogenization, heating in a boiling water bath for 15 minutes, and incubation for 40 hours with pancreatin in media supplemented with 5×10^{-3} M $MnCl_2 \cdot 4H_2O$ and 1×10^{-3} M thiouracil. After acidification of the hydrolyzate the radioiodine was extracted with butanol saturated with N/10 HCl. Butanol extracts were concentrated before application to Whatman No. 1 paper 16 by 16 inches for 2 dimensional chromatography. The solvent systems utilized were butanol : dioxane : ammonia[6] and butanol : acetic acid : water.[7] Addition of the carriers, potassium iodide, monoidotyrosine, diiodotyrosine, triiodothyronine, and thyroxine, permitted identification of these compounds. Radioactivity was found by means of radioautographs of the chromatograms on no-screen x-ray film. Ultraviolet absorbing areas on each chromatogram were located by placing the chromatogram over a sheet of Kodak photographic paper AD type A-4 and overlaying with a Corning No. 9863 ultraviolet transmitting filter. These were exposed for 1 second to a 15 watt fluorescent lamp. The paper was developed with Kodak Dektol developer, fixed, and washed in the usual manner. The areas of radioactivity and ultraviolet absorption, thus determined, were marked on the chromatogram. To provide a permanent record a contact print of the marked chromatogram was made. Then all the outlined areas were cut out and counted in a well-type scintillation counter. Radioactivity in each area was expressed as per cent I^{131} on the chromatogram.

RESULTS

Propylthiouracil at 10^{-3} to 10^{-5} M had an effect on in vitro metabolism of I^{131} by thyroid mince. As seen in Table I radioiodide uptake was depressed in both the presence and absence of thyrotropin. The effect of propylthiouracil was not limited to the uptake of iodine since this agent resulted in an abnormal

TABLE I. EFFECT OF N-PROPYLTHIOURACIL ON IODINE UPTAKE

PROPYLTHIOURACIL (MOLAR CONCENTRATION)	% UPTAKE TSH CONCENTRATION (MU./ML.)	
	0	20
0	37.3 ± 11.0	59.4 ± 13.8
10^{-3}	19.6 ± 6.7	29.4 ± 10.2
5×10^{-4}	22.6 ± 6.4	23.5 ± 5.1
10^{-4}	20.6 ± 4.1	26.8 ± 6.6
5×10^{-5}	19.9 ± 5.3	28.0 ± 6.7
10^{-5}	29.2 ± 5.8	42.3 ± 18.0
10^{-7}	45.4 ± 9.2	69.0 ± 11.5

distribution of the radioactive amino acids. One can readily compare the I^{131} utilization in the presence and absence of propylthiouracil in Figs. 1 and 2, radioautographs of mince untreated (Fig. 1) and after incubation in 10^{-3} M propylthiouracil (Fig. 2). Data in Table II indicate that synthesis of mono- and diiodotyrosines are depressed significantly by as little as 10^{-5} M propylthiouracil (monoiodotyrosine $p = 0.001$, diiodotyrosine $p = 0.005$). With this decrease in iodotyrosine synthesis was a corresponding increase in radioactivity found as iodide. Although propylthiouracil blocked iodination of tyrosine it did

not completely inhibit the iodine uptake response to thyroid stimulating hormone. In both the presence and absence of propylthiouracil thyroid stimulating hormone increased the I^{131} taken up as indicated in Table I.

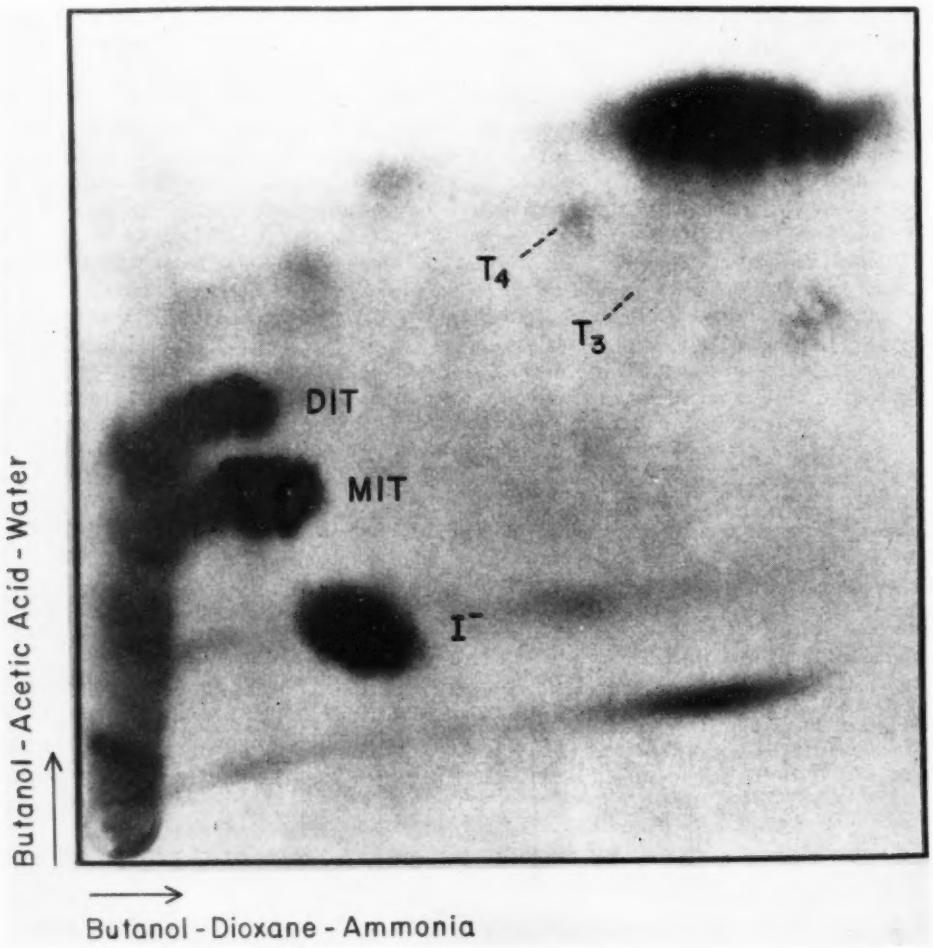

Fig. 1.—A radioautograph of a two dimensional chromatogram of an extract of beef thyroid tissue incubated with I^{131}.

Sodium perchlorate also exerted an effect on in vitro iodine metabolism. As seen in Table III iodine uptake was decreased at 10^{-3} and 10^{-4} M ($p < 0.001$). In spite of these changes in radioiodine uptake it appeared that thyrotropin exerted a stimulatory effect on uptake at all levels of perchlorate employed. Utilization of iodine was not greatly influenced by sodium perchlorate. Data in Table IV show that in the presence of sodium perchlorate mono- and diiodotyrosines and iodide were distributed in a pattern which was not statistically different from untreated tissue.

It is of interest to examine changes in the distribution of radioiodine induced by addition to thyrotropic hormone. The fraction of radioactivity

present as monoiodotyrosine was not significantly altered by thyroid stimulating hormone ($p = 0.200$). However labeled diiodotyrosine was increased in the presence of thyrotropin ($p = 0.030$), primarily at the expense of the iodide fraction.

TABLE II. EFFECT OF N-PROPYLTHIOURACIL ON DISTRIBUTION OF I^{131} IN THYROID

	PROPYLTHIOURACIL (MOLAR CONCENTRATION)	CONCENTRATION ON PAPER (% OF TOTAL I^{131}) TSH (MU./ML.)	
		0	20
Monoiodotyrosine	0	31.5 ± 6.8	35.7 ± 7.0
	10^{-3}	0.3 ± 0.4	0.1 ± 0.2
	5×10^{-4}	0.4 ± 0.6	1.0 ± 1.1
	10^{-4}	2.1 ± 1.3	2.5 ± 1.1
	5×10^{-5}	1.3 ± 0.9	1.6 ± 1.5
	10^{-5}	14.5 ± 4.5	18.7 ± 7.1
	10^{-7}	30.0 ± 5.4	33.6 ± 4.4
Diiodotyrosine	0	8.6 ± 5.1	14.9 ± 6.0
	10^{-3}	0.1 ± 0.1	0.1 ± 0.2
	5×10^{-4}	0.2 ± 0.1	0.1 ± 0.1
	10^{-4}	0.2 ± 0.1	0.2 ± 0.1
	5×10^{-5}	0.2 ± 0.2	0.9 ± 1.5
	10^{-5}	2.3 ± 1.8	4.4 ± 2.9
	10^{-7}	11.9 ± 3.3	20.3 ± 4.5
Iodide	0	21.4 ± 15.8	12.9 ± 7.7
	10^{-3}	70.6 ± 33.5	75.2 ± 34.6
	5×10^{-4}	93.1 ± 0.6	93.1 ± 2.2
	10^{-4}	93.1 ± 2.9	93.1 ± 2.7
	5×10^{-5}	92.4 ± 4.4	90.7 ± 5.0
	10^{-5}	58.9 ± 25.4	50.4 ± 21.3
	10^{-7}	18.8 ± 12.7	9.0 ± 6.6
Solvent front	0	17.9 ± 8.7	11.4 ± 6.0
	10^{-3}	22.9 ± 8.4	20.9 ± 12.1
	5×10^{-4}	1.9 ± 1.4	3.0 ± 1.4
	10^{-4}	2.1 ± 1.9	2.0 ± 1.2
	5×10^{-5}	2.5 ± 0.8	3.4 ± 2.6
	10^{-5}	15.9 ± 15.3	14.3 ± 12.6
	10^{-7}	19.7 ± 10.4	10.6 ± 6.0

TABLE III. EFFECT OF PERCHLORATE ON IODINE UPTAKE

PERCHLORATE (MOLAR CONCENTRATION)	UPTAKE % TSH CONCENTRATION (MU./ML.)	
	0	20
0	31.0 ± 5.3	63.1 ± 15.7
10^{-3}	13.1 ± 0.1	23.9 ± 3.0
10^{-4}	15.0 ± 0.8	30.2 ± 4.0
10^{-5}	26.8 ± 7.5	59.9 ± 21.3
10^{-7}	34.8 ± 7.8	71.1 ± 6.6

Accuracy of measurement of thyroxine and triiodothyronine is subject to some question. As seen in Fig. 3, a contact print of a chromatogram marked for radioactive and ultraviolet absorbing areas, these amino acids travel very close to the solvent front material whose radioactivity is comparatively high. The possibility of some of the latter compound trailing into the T_3 T_4 areas cannot be eliminated. In addition radioactivity of T_3 and T_4 is relatively low. Because of these limitations, concentrations observed for thyroxine and triiodothyronine have not been reported.

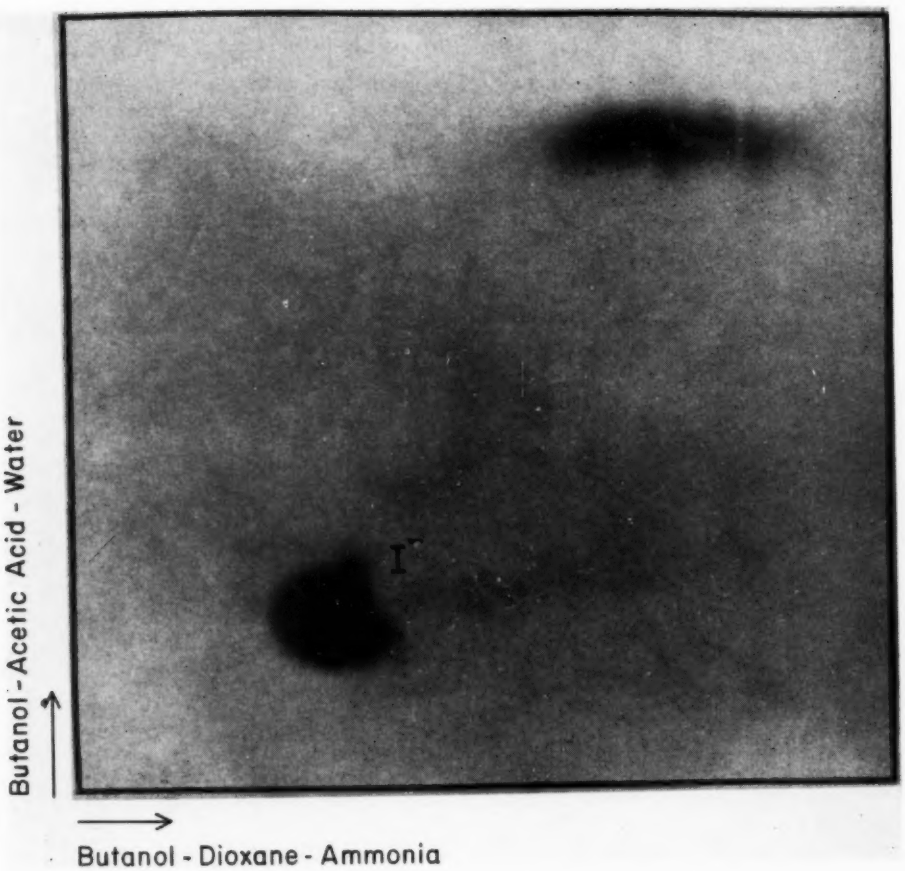

Fig. 2.—A radioautograph of a two dimensional chromatogram of an extract of beef thyroid tissue which had been incubated with radioactive iodine in the presence of 10^{-3} M propylthiouracil.

DISCUSSION

The inhibitory effect of 6-propylthiouracil on halogenation of tyrosine and monoiodotyrosine has been observed in the rat.[2, 4, 8] In the study reported here similar observations were made with minces of beef thyroid with respect to direction of change of the ratio of monoiodotyrosine to diiodotyrosine (MIT/DIT). There was a ratio of monoiodotyrosine to diiodotyrosine of 3.7 in untreated mince and 3.0 in mince incubated in 10^{-3} M propylthiouracil. That this compound blocks the iodination of monoiodotyrosine more than its synthesis is apparent from the ratios of tissue incubated in propylthiouracil at levels that affected iodine uptake 10^{-3} to 10^{-5} M. These ratios are in Table V. The magnitude of the ratio of monoiodotyrosine to diiodotyrosine of untreated mince in the in vitro system is more comparable to 3.4 reported by Tong and Chaikoff[9] for thyroid mitochondria than to the figure 0.62 of Richards and Ingbar[2] or 0.48 of Slingerland[4] for tyrosines synthesized in vivo in the rat. The higher ratios may represent species difference or the effects of the in vitro system on iodine metabolism.

In spite of these nonphysiologic conditions propylthiouracil interfered primarily with synthesis of mono- and diiodotyrosines. It would appear that there is some difference in the enzymes associated with halogenations of tyrosine. In this laboratory it was observed[10] that there is an abnormal ratio of radioactive amino acids synthesized in beef thyroid mince, similarly incubated in 2, 4-dinitrophenol. The latter agent permitted monoiodotyrosine to form but blocked its conversion to diiodotyrosine, suggesting that the introduction of the second iodine atom to the tyrosine molecule is associated with oxidative phosphorylation.

I^{131} taken up by thyroid tissue in the presence of propylthiouracil was found principally in the form of iodide. This does not eliminate the possible oxidation of iodide with production of a compound analogous to unknown-2 of Taurog and co-workers.[11] These investigators reported the unknown to

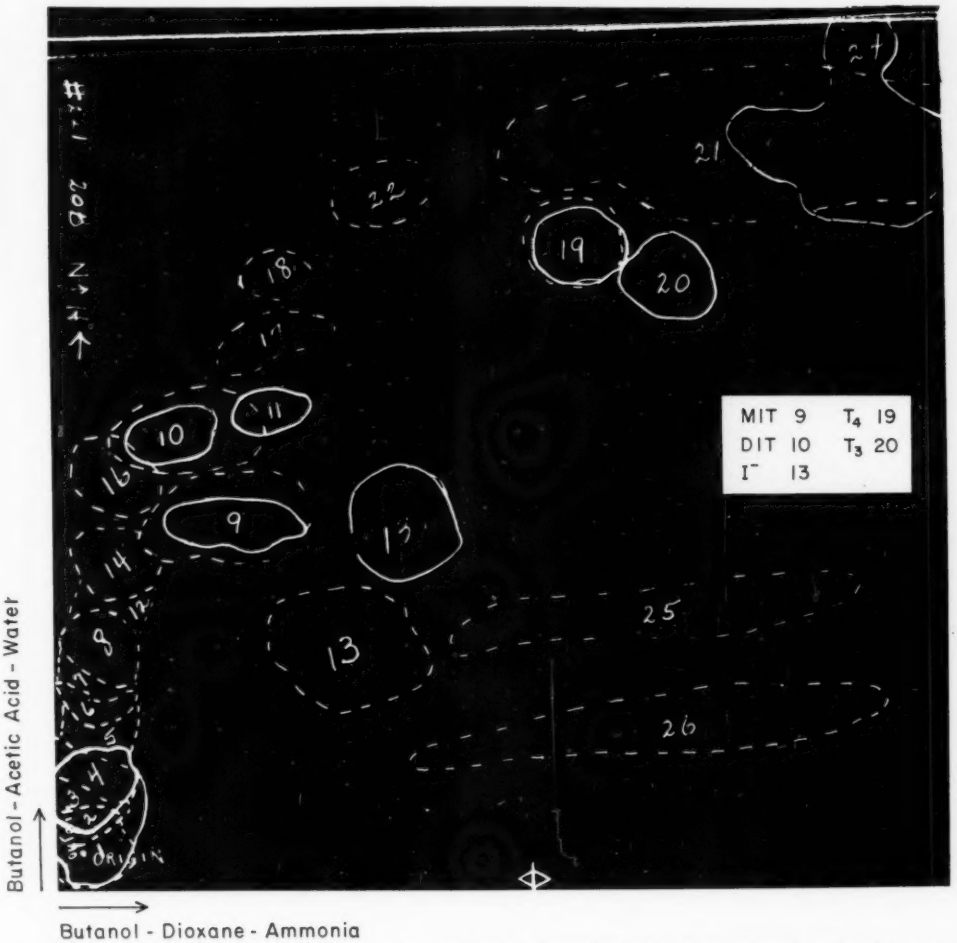

Fig. 3.—A contact print of a two dimensional chromatogram of an extract of beef thyroid tissue incubated with I^{131}. The areas of radioactivity as indicated by a radioautograph of this chromatogram are represented by the broken line areas. The solid line areas indicate the location of ultraviolet absorbing material. The identity of certain of these numbered areas is indicated in the square.

TABLE IV. EFFECT OF PERCHLORATE ON DISTRIBUTION OF I^{131} IN THYROID

	PERCHLORATE (MOLAR CONCENTRATION)	CONCENTRATION ON PAPER (% OF TOTAL I^{131}) TSH (MU./ML.)	
		0	20
Monoiodotyrosine	0	32.7 ± 6.7	38.8 ± 6.7
	10^{-3}	40.1 ± 3.9	35.2 ± 7.7
	10^{-4}	38.0 ± 3.2	39.4 ± 5.2
	10^{-5}	34.4 ± 9.7	34.1 ± 8.4
	10^{-7}	38.4 ± 7.3	44.9 ± 3.5
Diiodotyrosine	0	3.9 ± 1.9	13.0 ± 5.7
	10^{-3}	2.6 ± 0.9	8.2 ± 7.4
	10^{-4}	4.2 ± 1.5	9.3 ± 4.9
	10^{-5}	5.0 ± 2.2	11.7 ± 5.6
	10^{-7}	5.9 ± 0.1	15.9 ± 5.5
Iodide	0	31.4 ± 7.0	16.3 ± 7.7
	10^{-3}	30.5 ± 13.3	20.1 ± 5.2
	10^{-4}	26.7 ± 3.6	21.3 ± 4.0
	10^{-5}	30.1 ± 15.2	14.4 ± 5.6
	10^{-7}	23.9 ± 20.6	7.4 ± 2.3
Solvent front	0	7.8 ± 4.1	6.2 ± 1.0
	10^{-3}	18.6 ± 4.3	13.9 ± 4.4
	10^{-4}	15.9 ± 5.4	9.7 ± 0.6
	10^{-5}	10.8 ± 6.4	4.6 ± 2.7
	10^{-7}	13.3 ± 5.3	4.6 ± 0.1

TABLE V. EFFECT OF PROPYLTHIOURACIL ON RATIO OF MONOIODOTYROSINE TO DIIODOTYROSINE

PROPYLTHIOURACIL (MOLAR CONCENTRATION)	MIT/DIT
0	3.7
10^{-3}	3.0
5×10^{-4}	2.0
10^{-4}	10.5
5×10^{-5}	6.5
10^{-5}	6.3
10^{-7}	2.5

yield mainly iodide after its incubation in a thyroid particulate fraction supplemented with pancreatin. This might also be the source of the I^{131} released from the thyroid of humans maintained on propylthiouracil after administration of thiocyanate.[12] It should be noted that propylthiouracil only partially inhibits the stimulatory action of thyrotropin on total I^{131} uptake. Radioiodine uptake in the presence of propylthiouracil and thyroid stimulating hormone approaches that of untreated mince. However the I^{131} in such tissue was principally in the form of iodide. These data indicate that even in the presence of thyrotropin, propylthiouracil induces alterations in thyroidal iodine synthesis which yield little iodotyrosines and thyronines and much physiologically inactive iodide.

SUMMARY

1. Graded doses of propylthiouracil, when incubated with a beef thyroid mince preparation in a radioiodine supplemented media, resulted in a decrease in I^{131} uptake.

2. Iodotyrosine synthesis was markedly inhibited in the presence of propylthiouracil. The iodination of monoiodotyrosine was particularly sensitive to this drug.

3. Sodium perchlorate was capable of marked diminution of the iodine uptake of the mince preparation but was without effect on the distribution of iodotyrosines.

REFERENCES

1. Tong, W., Taurog, A., and Chaikoff, I. L.: Activation of the Iodinating System in Sheep Thyroid Particulate Fractions by Flavin Cofactors, J. Biol. Chem. **227**: 773, 1957.
2. Richards, J. B., and Ingbar, S. H.: The Effects of Propylthiouracil and Perchlorate on the Biogenesis of Thyroid Hormone, Endocrinology **65**: 198, 1959.
3. Anbar, M., Guttmann, S., and Lewitus, Z.: The Mode of Action of Perchlorate Ions on the Iodine Uptake of the Thyroid Gland, Internat. J. Appl. Radiation **7**: 87, 1959.
4. Slingerland, D. W., Graham, D. E., Josephs, R. K., Mulvey, P. F., Jr., Trakas, A. P., and Yamazaki, E.: The Effect of Propylthiouracil on the Conversion of Monoiodotyrosine to Diiodotyrosine, Endocrinology **65**: 178, 1959.
5. Hart, K. T., Druet, D., and Mack, R. E.: Quantitative in Vitro Response of Thyroid Tissue to Thyrotropic Hormone, Endocrinology **64**: 857, 1959.
6. Gross, J., Leblond, C. P., Franklin, A. E., and Quastel, J. H.: Presence of Iodinated Amino Acids in Unhydrolyzed Thyroid and Plasma, Science **111**: 605, 1950.
7. Roche, J., Jutisz, M., Lissitzky, S., and Michel, R.: Chromatographie qualitatif des acides amines iodes radioactifs de la thyroglobuline marquée, Biochim. et biophys. acta **7**: 257, 1951.
8. Iino, S., and Yamada, T.: Effect of Graded Doses of Propylthiouracil (PTU) on Biosynthesis of Thyroid Hormone, Fed. Proc. **19**: 172, 1960.
9. Tong, W., and Chaikoff, I. L.: Formation of Iodotyrosines in Thyroid Mitochondria and Microsomes, Fed. Proc. **19**: 171, 1960.
10. Hart, K. T., Bauer, M. A., Druet, D., and Mack, R. E.: Effect of 2, 4-Dinitrophenol on Iodine Metabolism. In preparation.
11. Taurog, A., Tong, W., and Chaikoff, I. L.: An Unidentified Iodine Compound Formed by Incubation of Cell-Free Preparations of Tissue with Iodide-I^{131}, J. Biol. Chem. **227**: 759, 1957.
12. Stanley, M. M., and Astwood, E. B.: The Accumulation of Radioactive Iodide by the Thyroid Gland in Normal and Thyrotoxic Subjects and the Effect of Thiocyanate on Its Discharge, Endocrinology **42**: 107, 1948.

EVALUATION OF TRISODIUM CALCIUM DIETHYLENETRIAMINEPENTAACETATE IN IRON STORAGE DISEASE

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WITH THE TECHNICAL ASSISTANCE OF KENNETH MCCOY AND EDWARD J. ZAPOLSKI

The results of parenteral administration of the chelate trisodium calcium diethylenetriaminepentaacetate (DTPA) in five patients with iron storage disease are reported. DTPA given intravenously in doses of 2.5 to 4.0 Gm. resulted in iron excretion of up to 109 mg. per 24 hours in three patients with clinical hemochromatosis. Two control subjects showed no increase in urinary iron after the chelate was given. There was little, if any, clinical toxicity, and no significant effect on hemogram, urinalyses, blood urea nitrogen, or serum calcium. Prothrombin times were only slightly prolonged. Comparable iron excretion occurred after intravenous and intramuscular administration of DTPA. The amount of iron excreted in various subjects correlated best with the estimated iron stores. The hypothesis is advanced that parenterally administered DTPA is carried in the circulation to iron storage sites, the calcium in the chelate is exchanged for iron, and this complex continues in the circulation and is excreted in the urine. The increased efficiency of DTPA over EDTA is discussed. The clinical application of DTPA is not established but the results reported here suggest a possible role as a supplement to phlebotomy in the treatment of hemochromatosis, and as an alternative therapy for individuals with iron storage disease not suitable for phlebotomy.

IDIOPATHIC hemochromatosis and transfusion hemosiderosis have in common the presence of iron excess in the body. This is predominantly storage iron, deposited in the parenchyma of many organ systems. Tissue damage, manifested by altered organ function and fibrosis, eventually occurs and increases with the passage of time. The development of this concept has led to therapeutic removal of iron from the body in an attempt to arrest or reverse the course of iron storage disease. The most effective method of iron removal is by repeated phlebotomy. This was first reported by Balfour and associates[1] and has since become accepted as the treatment of choice in most cases of idiopathic hemochromatosis.[2, 3] However, in some patients repeated phlebotomies may be impractical. Anemia with advanced liver disease, coronary insufficiency, or other considerations may weigh against this therapy. Transfusion hemosiderosis, usually associated with severe anemia, presents the same problem. Alternative approaches have therefore been explored.

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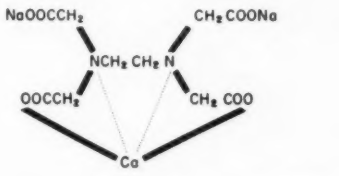
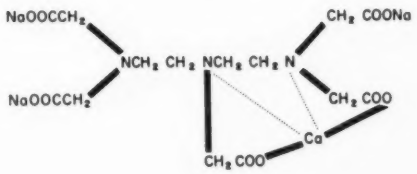
Chelation offers a theoretically feasible method of treatment for iron storage disease, and would be especially suited to the complications just mentioned. By this approach the chelating compound is introduced into the body where it forms an iron-chelate complex. This complex is then excreted in the urine. Foreman and co-workers[4] reported that the urinary excretion of iron in rats can be increased by the intraperitoneal administration of such a chelate (Fe-3-specific). However, clinical experience with chelates to date has been disappointing. Ohlsson and collaborators[5] obtained less than 3 mg. of iron per 24 hours in urine after British anti-lewisite therapy. Others[6-11] using EDTA and related compounds have recovered up to 10.5 mg. of iron per 24 hours when the agent was administered intravenously. In contrast, about 250 mg. of iron is removed in a single phlebotomy.

The search for more effective iron chelates has been guided by several biochemical and physiologic considerations: (1) no known chelate will remove iron from the plasma iron-binding protein (IBP) at pH 7.4[12]; (2) the iron-chelate bond must be strong enough to be maintained in the presence of the competing plasma iron-binding protein; (3) the iron-chelate complex should be completely excreted; (4) the chelate should be nontoxic.



DISODIUM CALCIUM ETHYLENE DIAMINE TETRAACETATE

(EDTA)



TRISODIUM CALCIUM DIETHYLENETRIAMINE PENTA ACETATE

(DTPA)

Fig. 1.—Comparison of chemical structure of EDTA and DTPA.

An agent structurally similar to EDTA, trisodium calcium diethylenetriaminepentaacetate (DTPA), is shown in Fig. 1. It does not yield iron to the plasma iron-binding protein in vitro at pH 7.4.[12] Excretion of the iron-DTPA complex is essentially quantitative in rats after parenteral administration. In comparison the excretion of iron EDTA is approximately 70 per cent under the same circumstances.[13] Finally, animal studies with DTPA have revealed no gross or microscopic toxicity.[13] This evidence suggested that DTPA might be effective in mobilizing iron in humans. Clinical trials were then undertaken, and this article constitutes the first report on the use of DTPA as a chelate in iron storage disease.

TABLE I. CLINICAL DATA ON PATIENTS WITH

PATIENT	AGE (YR.)	RACE	SEX	DIAGNOSIS	PHYSICAL FINDINGS	HCT (%)	SERUM IRON AND UIBC (μG/100 ML.)
W. D.*	50	W	M	Hemochromatosis Arteriosclerotic heart disease	Hepatomegaly, ascites, pedal edema	37	Fe 210 UIBC 0
P. H.	58	W	M	Hemochromatosis Esophageal varices	Bronzed skin, hepatosplenomegaly	43	Fe 202 UIBC 0
P. M.	45	W	F	Thalassemia major Transfusion hemochromatosis Recurrent pulmonary embolization	Bronzed skin, hepatomegaly	33	Fe 190 UIBC 0
R. D.*	43	W	M	No clinical disease	Normal	47	Fe 238 UIBC 0
H. D.*	48	W	M	No clinical disease	Normal	43	Fe 158 UIBC 100

*Siblings.

†The numerator in this fraction represents estimated iron stores, the denominator the maximum grade. Most normal subjects have Gr 2/6 to 3/6 iron stores.

‡Diagnosis in one sibling not proved by biopsy.

METHODS AND MATERIALS

Five subjects with varying degrees of iron excess demonstrated by liver biopsy and aspiration bone marrow were studied. Observations were also made on two individuals without increased iron.

Each patient had a thorough history and physical examination with appropriate clinical laboratory studies. Serum and urine levels were done by the method of Ramsay,[14] slightly modified as follows: The reagent used consisted of 500 mg. 222 tripyridine, 500 mg. hydroxylamine HCl, 23.52 ml. 36 per cent acetic acid, and 34.12 Gm. sodium acetate hydrate (National Formulary granular). The mixture is brought to 500 ml. with iron-free water, mixed well, and heated at 50 to 70° C. until a drop of oily reagent is formed at the bottom of the flask. The supernatant is then decanted into an iron-free flask and stored in the dark at room temperature. It is stable for at least 3 to 4 weeks. Ramsay's improved technique is then followed.[14a] Protein precipitated by heating the serum samples is removed by centrifugation at 3,500 r.p.m. for 15 minutes. The same procedure is followed for the urine iron determinations with two further modifications: (a) tubes containing the urine samples and reagent mixture are heated in a boiling water bath for 15 minutes instead of 5 to 10 minutes; (b) urine turbidity varies, and is difficult to eliminate without removing the iron in the sample at the same time. Therefore, the unknown sample is read against a water blank in the Coleman Junior spectrophotometer at 520 mμ and from this reading both a reagent blank and a "urine blank" (urine sample plus reagent mixture less tripyridine) are subtracted.

The unsaturated iron-binding capacity (UIBC) of serum was determined by the method of Rath and Finch.[15] Body iron stores were estimated by aspiration bone

WITH IRON STORAGE DISEASE

LIVER FUNCTION TESTS	GLUCOSE TOLERANCE TEST	LIVER BIOPSY	IRON STORES		REMARKS
			LIVER	MARROW	
BSP 16% Alkalin phosphatase 3.6 Bessey-Lowry units Albumin 4.0 Gm./100 ml. Globulin 2.7 Gm./100 ml.	Abnormal	Hemochromatosis	Extensive parenchymal deposition	Gr 5/6†	2 Siblings with hemochromatosis‡
BSP 10% Alkalin phosphatise 3.7 Bessey-Lowry units Albumin 3.0 Gm./100 ml. Globulin 4.2 Gm./100 ml.	Abnormal	Hemochromatosis	Extensive parenchymal deposition	Gr 4/6	1 Sibling with hemochromatosis
BSP 0 Alkalin phosphatase 2.0 Bessey-Lowry units Albumin 3.1 Gm./100 ml. Globulin 4.8 Gm./100 ml.	Clinical diabetes (70u PZI)	Hemochromatosis	Extensive parenchymal deposition	Gr 6/6	100+ transfusions splenectomy, 1948
BSP 2% Alkalin phosphatase 1.7 Bessey-Lowry units Albumin 4.6 Gm./100 ml. Globulin 2.6 Gm./100 ml.	Abnormal	Hemosiderosis	Moderate parenchymal deposition	Gr 4+/6	3 siblings with hemochromatosis
BSP Trace Alkalin phosphatase 2.0 Bessey-Lowry units Albumin 4.4 Gm./100 ml. Globulin 2.8 Gm./100 ml.	Abnormal	Hemosiderosis	Moderate parenchymal deposition	Gr 4/6	3 siblings with hemochromatosis

marrow[16] in all cases and by liver biopsy in all cases but two. DTPA* was administered intravenously over a 3 hour period as a 25 per cent solution of the trisodium monocalcium salt diluted in 500 ml. 5 per cent dextrose in water. The chelate was given intramuscularly as a 25 per cent solution without further dilution. Twenty-four hour urine collections were made in iron-free equipment.

RESULTS

Table I lists the essential clinical data on the patients in this study. For purposes of this discussion, hemochromatosis is defined as extensive intracellular iron deposition with fibrosis in the liver. The term hemosiderosis refers to iron deposition, intracellular or otherwise, without fibrosis or other evidence of structural damage to the liver.

W. D., a 50-year-old man, had advanced arteriosclerotic heart disease in addition to hemochromatosis. Phlebotomy was therefore considered hazardous. Four separate infusions of DTPA were given, with the results shown in Fig. 2, *A*. Control urine collections yielded less than 1.0 mg. of iron daily. In the 24 hours after administration of 2.5 Gm. of DTPA, 42.0 mg. of iron was excreted in the urine, with return to control levels the following day. Daily infusions of 4.0 Gm. DTPA were then given for 3 days. The initial 24 hour excretion was

*DTPA used in this study was supplied through the courtesy of Dr. M. Weiner, Pharmaceutical Division, Geigy Chemical Corporation, Ardsley, N. Y.

quite high (109.2 mg.), but urine iron content fell off sharply on the two subsequent days to 45.9 and 27.5 mg., respectively. Serum iron levels were in general unaffected with the exception of the third 4.0 Gm. infusion day, when the preinfusion value was elevated to 490 μg per 100 ml. The serum iron-binding protein was fully saturated on all occasions.

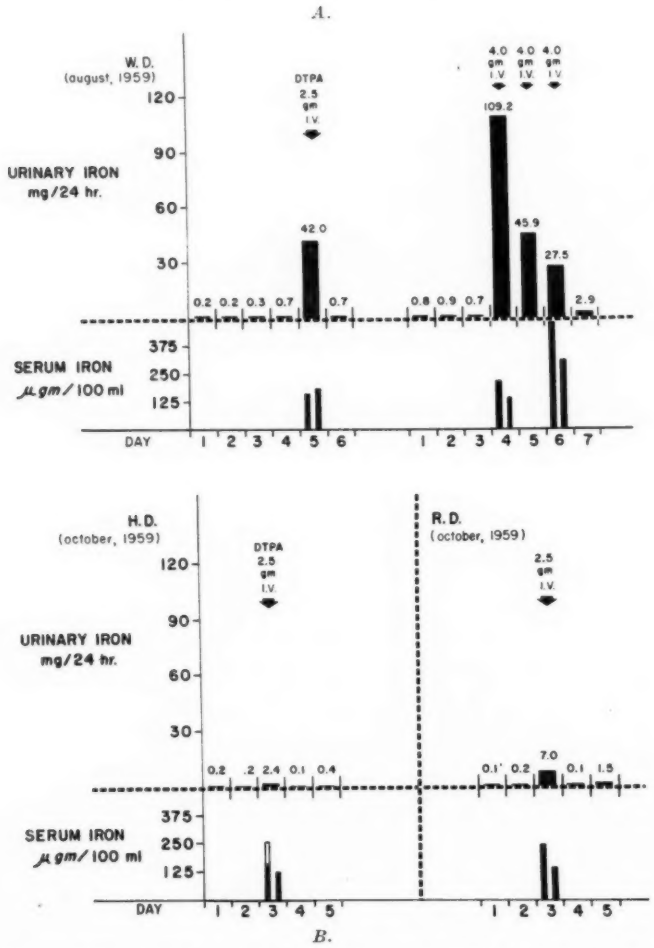


Fig. 2.—Urinary iron excretion and serum iron after DTPA administration in three siblings: W. D., H. D., and R. D. The iron-binding protein of the serum was completely saturated in all instances but one. In this one determination (H. D., preinfusion) the unsaturated iron-binding protein is represented by an open column above the serum iron level.

R. D. (age 43 years) and H. D. (age 48 years) were asymptomatic male siblings of W. D. Although both of these subjects were normal on physical examination and both had normal liver function tests, the glucose tolerance tests were in the abnormal range and liver biopsies revealed moderate intracellular iron deposition without fibrotic changes (Table I). Iron excretion data are shown in Fig. 2, *B*. H. D. was the only patient studied who had an unsaturated serum iron-binding protein. He also had somewhat less marrow iron than his brother, R. D., and he excreted less iron after the same dose of chelate.

P. H. was a 58-year-old man with hemochromatosis who was undergoing a course of weekly phlebotomies. At the time of study approximately 3.7 Gm. of iron had been removed with 15 pints of blood. A DTPA infusion was given on the day after a phlebotomy to determine whether iron was available for chelation under these circumstances. The results are shown in Fig. 3. In the 24 hours after infusion of 2.5 Gm. of DTPA, 22.4 mg. of iron was excreted.

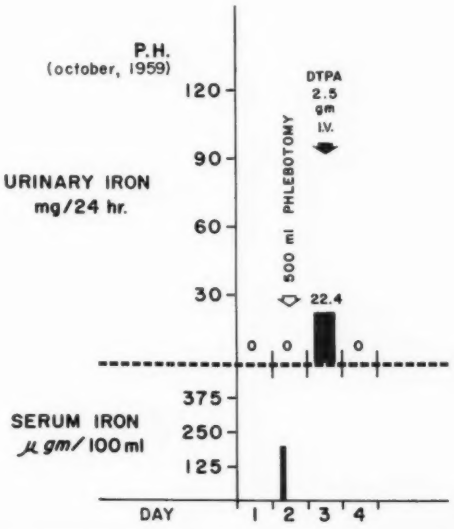


Fig. 3.—Urinary iron excretion and serum iron in a patient with hemochromatosis who was undergoing phlebotomy.

Patient P. M., a 45-year-old woman with thalassemia major, represented a different problem. She had received over 100 transfusions in a 20 year period and had developed a clinical picture indistinguishable from idiopathic hemochromatosis. Liver biopsy showed parenchymal iron deposition and fibrosis. Phlebotomy was contraindicated because of the persistent hemolytic anemia with an average hematocrit of 33 per cent. Four infusions of DTPA were given as shown in Fig. 4. The first dose of 2.5 Gm. resulted in a 24 hour urine iron excretion of 30.4 mg. Subsequent infusions, spaced as shown, resulted in greater excretions, with a maximum of 66.8 mg. of iron removed after the final 2.5 Gm. of chelate.

This patient was studied further to determine the effect of various doses of intravenous chelate. Fig. 5 shows iron excretion after DTPA doses ranging from 0.5 to 3.0 Gm. Iron excretion tended to increase as the dose of chelate was raised from 0.5 to 1.0 Gm. However, there was considerable variation in the amount of iron obtained with both the 1.0 and the 2.5 Gm. infusions. Higher doses of DTPA (2.5 and 3.0 Gm.) did not result in uniformly increased amounts of urinary iron.

All of these subjects had increased iron stores to some degree. In contrast, a normal subject with normal marrow iron stores (Gr 3/6)* and a nonanemic

*Marrow iron is graded 0/6 to 6/6. Stores in most normal subjects are 2/6 to 3/6.

subject with low iron stores (Gr 0/6) were given 2.5 Gm. of DTPA. The results are shown in Fig. 6. Iron excretion was not increased by the chelate in either case.

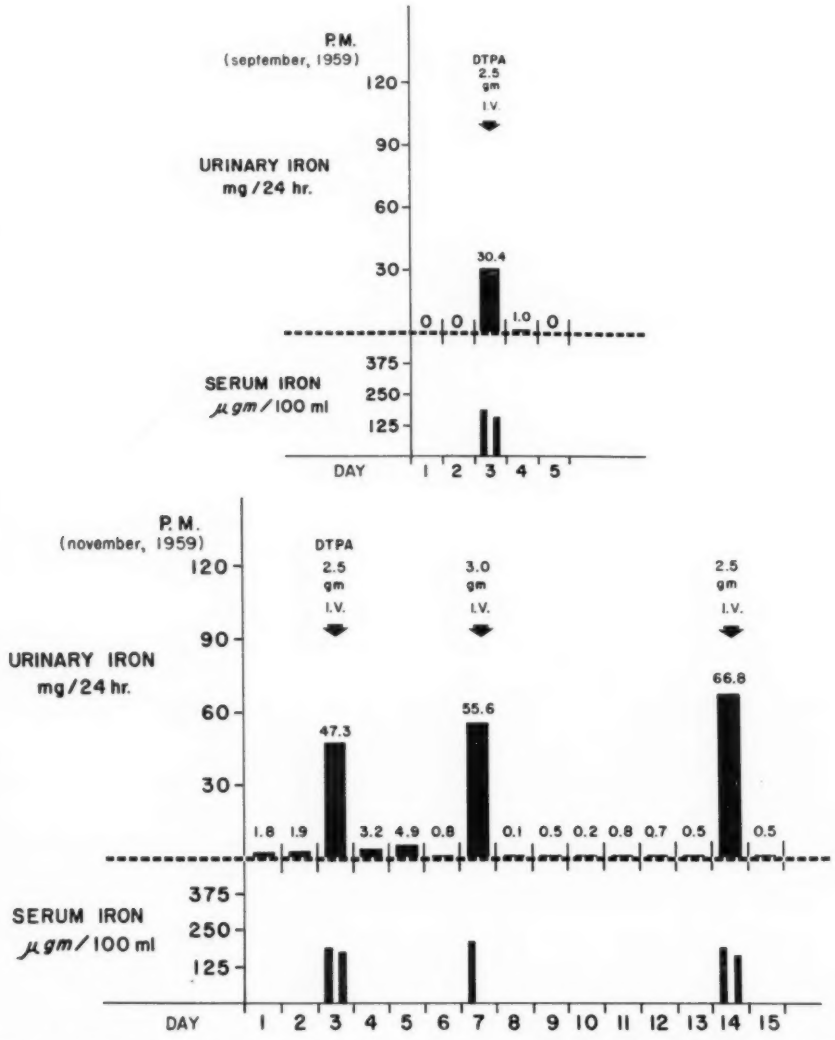


Fig. 4.—Urinary iron excretion and serum iron after administration of DTPA in a patient with transfusion hemochromatosis.

Of interest was the time when the maximum amount of iron appeared in the urine after administration of the chelate. Fig. 7 illustrates urine iron content at various intervals after an intravenous infusion of 2.5 Gm. DTPA. The total excretion (patient W. D.) in 24 hours was 42.0 mg., the major portion (36.6 mg.) being removed in the first 12 hours.

Toxic reactions to the drug were mild. Nausea, vomiting, and mild diarrhea were encountered intermittently in two patients, but these symptoms could not

be definitely related to DTPA. Blood counts, urinalyses, and blood urea nitrogen levels were unaffected. Changes in prothrombin time (Quick) and serum calcium before and after intravenous chelate are shown in Fig. 8. Although the DTPA was given as the monocalcium salt, there was no consistent

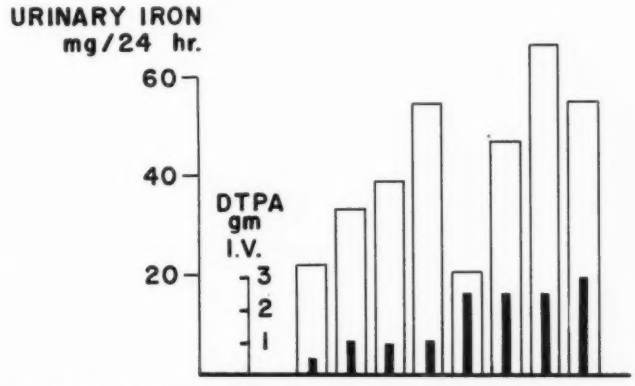


Fig. 5.—Iron excretion in subject P. M. after varying intravenous doses of DTPA. The solid column represents the DTPA dosage in grams. The open column indicates the 24 hour urinary iron excretion.

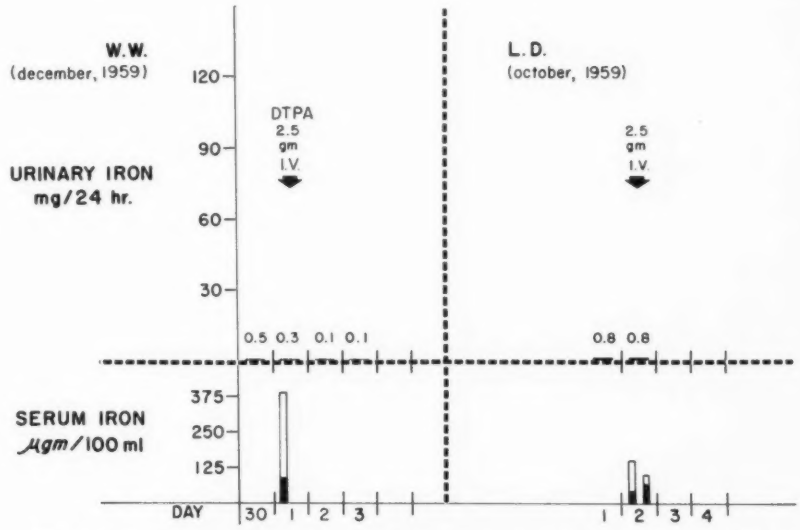


Fig. 6.—The upper portion illustrates iron excretion in two subjects without iron excess. The solid portion of the columns on the lower part of the figure represents the serum iron, and the open columns indicate the unsaturated iron binding capacity.

effect on serum calcium. The greatest change (a rise from 10.4 to 13.2 mg. per 100 ml.) occurred after a 4 Gm. infusion of DTPA. This was the largest amount of chelate given in a 24 hour period. Prothrombin times were in general increased after DTPA but the change never exceeded 2 seconds even when the initial value was abnormal.

In another experiment smaller doses of DTPA were given intramuscularly and intravenously with the results shown in Fig. 9. Fig. 9, *A*, illustrates

comparative iron excretion after equal doses of DTPA given by both routes. An intramuscular injection of 0.5 Gm. of the chelate resulted in the excretion of 22.0 mg. of urinary iron and essentially the same amount (22.1 mg.) was excreted after 0.5 Gm. intravenously. When the dose was raised to 1.0 Gm., iron

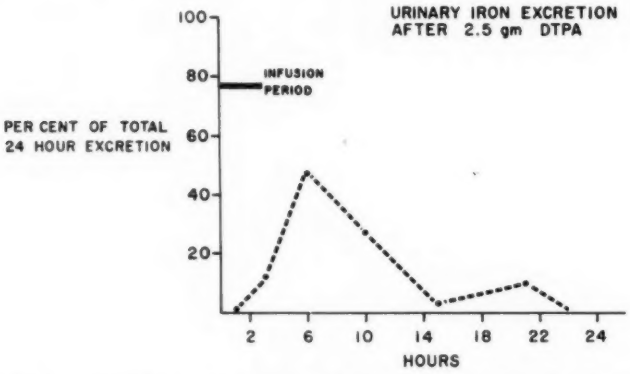


Fig. 7.—Urinary iron excretion plotted against time in a subject who excreted 42 mg. of iron in 24 hours. The major portion of the iron was excreted in the first 12 hours after administration of DTPA.

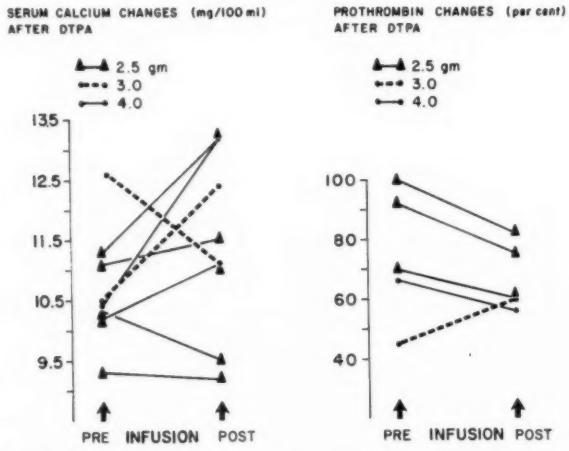


Fig. 8.—Pre- and postinfusion levels of calcium and prothrombin after varying intravenous doses of DTPA.

excretion increased to 39.5 mg. after intramuscular administration of DTPA and 54.2 mg. after intravenous administration of DTPA. The chelate was then given on alternate days for a series of 9 injections of 1.0 Gm. each, with two of the doses being given intravenously and the remainder intramuscularly. The results are shown in Fig. 9, *B*. Equivalent amounts of urinary iron were found after both routes of administration and there was no indication of a fall-off in iron excretion after repeated doses of the chelate. In this experiment urinary iron totaled 461 mg. on 13 injection days. The total amount of DTPA given in this period was 12 Gm. The fasting serum iron usually ranged between 200 and 400 μg per 100 ml., but on one occasion this value reached 748 μg per 100

ml. The iron-binding protein was completely saturated on all determinations. There was no persistent local reaction to the intramuscular injections. The pain with these injections was considered by the patient to be equivalent to that experienced with intramuscular procaine penicillin. On several injection days the passage of 2 to 3 soft stools was noted, but there was

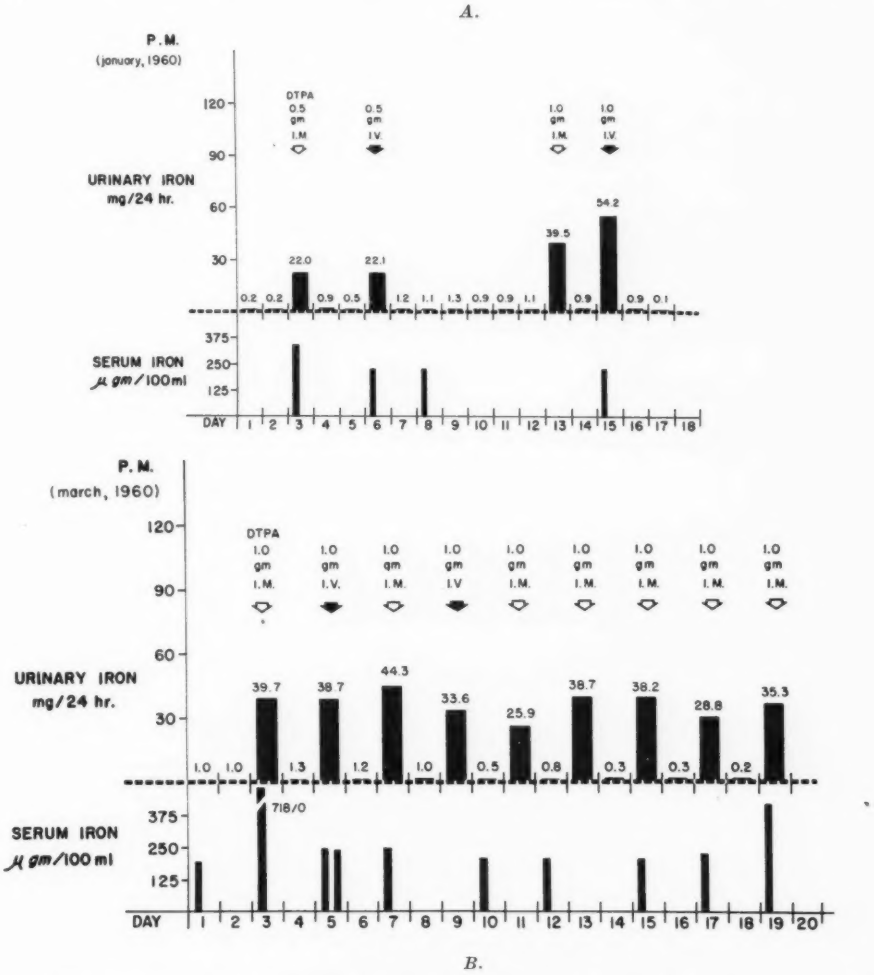


Fig. 9.—Urine and serum iron in subject P. M. after a series of intramuscular and intravenous doses of DTPA. Excretion of iron was roughly equivalent when the same dose of DTPA was given by both routes. The lower portion illustrates continued high urinary iron content after repeated injections of DTPA.

no frank diarrhea. No other toxic manifestations were observed. The patient continued to take her usual dose of Dicumarol throughout the injection period, and prothrombin times remained in the therapeutic range on most occasions, varying between 19 and 30 per cent of normal. Serum calcium ranged between 9.9 and 11.4 mg. per 100 ml. Peripheral blood, blood urea nitrogen, and urinalyses showed no change.

DISCUSSION

These data indicate that significant amounts of iron can be removed from the body by chelation with DTPA. Two hundred and twenty-five milligrams of iron was removed from patient W. D. with 4 separate doses of DTPA totaling 14.5 Gm. Over a longer period, 17 doses totaling 22.5 Gm. resulted in the removal of 660.7 mg. of iron from patient P. M. These figures compare well with the 250 mg. of iron removed with one pint of blood and are much greater than the results reported to date with other chelating agents. Although there was a fall-off in the level of urinary iron in patient W. D. after repeated daily infusions (Fig. 2), when DTPA was given on alternate days to patient P. M. (Fig. 8) iron excretion continued to be high throughout a course of 9 injections. In the latter patient iron continued to be available for chelation even after a significant amount had been removed. In addition, there was neither local nor systemic toxicity observed after repeated intramuscular injections of DTPA.

Although the exact mechanism of iron removal by chelation is unknown, several sources of iron may be considered in the light of current knowledge. The 3 to 5 Gm. of iron in the normal individual is distributed in several sites: (1) 2.0 to 2.5 Gm., or the bulk of the iron, is in the hemoglobin molecule of circulating red cells and a lesser amount (0.13 Gm.) is in myoglobin; (2) a small portion of the iron (0.003 Gm.) is in combination with the iron-binding protein (transferrin) of plasma; (3) a small (0.008 Gm.) but very important amount of iron is contained in every cell of the body in heme enzymes; (4) approximately 1 Gm. of iron is located in the body stores, chiefly in the reticuloendothelial cells of the liver and spleen.

Circulating hemoglobin is unlikely as a source of iron excretion. Red cells would presumably be destroyed in the process of removing their iron, and there was no evidence of hemolysis in any patient after administration of DTPA. Total myoglobin iron is not present in the body in amounts equal to the iron excretion achieved in some of the subjects, and thus could not be the source for chelation. In the patients studied there was no correlation between iron excretion after DTPA administration and the serum iron level. Furthermore, DTPA will not remove iron from the iron-binding protein in vitro at pH 7.4 and it seems unlikely that it would do so in vivo.

Beutler[17] has shown that at least one iron containing enzyme (cytochrome C in rat liver and kidney) may be depleted in iron deficiency before significant anemia develops. Enzyme determinations were not made in our patients but there were no symptoms that suggested depletion of iron containing enzymes. More important, since the total amount of body iron in enzymes is estimated to be 0.008 Gm., this could not be the source of the 30 mg. and more of iron that appeared in the urine after a single 2.5 Gm. injection of DTPA.

It seems most likely that storage iron is the source of the iron excreted since it was in this area that the subjects differed most. The induced iron excretion varied directly in proportion to the estimated iron stores. Comparing the effects of the initial 2.5 Gm. dose of DTPA, the normal subject and the nonanemic iron-deficient subject showed no increase in iron excretion. Patients

with moderate increases in iron stores had modest increases in iron excretion (2.4 and 7.0 mg.), and the patient with the most marked increase in iron stores excreted 42.0 mg. of iron. This relationship is illustrated in Table II.

TABLE II. CORRELATION OF IRON STORES WITH IRON EXCRETION

PATIENT	ESTIMATED BODY IRON STORES	IRON EXCRETION AFTER INITIAL 2.5 GM. DTPA (MG.)
W. D.*	4+	42.0
P. M.	4+	30.4
P. H.	3+	22.4
R. D.*	2+	7.0
H. D.*	2+	2.4
W. W.	1+	0.3
L. D.	0	0.8

*Siblings.

On the basis of these clinical observations, the following hypothesis is suggested: parenterally administered chelate is carried in the circulation to iron storage sites. Here, the calcium in the chelate is replaced by iron. This newly formed iron chelate complex continues in the circulation until it reaches the kidneys and is then excreted in the urine. Free filtration at the glomerulus is assumed because of the small molecular size of DTPA (molecular weight 497).

In this study, DTPA administration has resulted in far greater iron excretion than that obtained with comparable doses of EDTA. The explanation for this may lie in two important biochemical differences between them. First, their relative affinity for iron differs. The absolute value of the iron-binding strength of DTPA has been listed at $10^{28.6}$ in comparison to $10^{25.1}$ for EDTA.[18]

TABLE III. COMPARATIVE METAL BINDING OF EDTA AND DTPA

METAL ION		STABILITY CONSTANT (LOG) EDTA	DTPA
Magnesium	(Mg++)	8.69	9.02
Calcium	(Ca++)	10.59	10.11
Strontium	(Sr++)	8.63	9.68
Manganese	(Mn++)	14.04	15.11
Iron	(Fe+++)	25.10	28.60
Cobalt	(Co++)	16.31	19.00
Nickel	(Ni++)	18.62	20.21

Data taken from Chaberek and Martell[18]. The affinity of the chelating agent for the respective metal ions increases with the stability constant.

Second, it has been previously noted[12] that when Fe^{59} combined with chelate was administered parenterally to animals, urine iron recovery was only about 70 per cent of the injected dose with EDTA, in contrast to 90 to 100 per cent recovery with DTPA. Thus Fe^{59} EDTA may lose some of its attached iron in passage through the animal, resulting in less efficient iron excretion. It is also possible that a given amount of DTPA would actually chelate more iron from body stores than would the same amount of EDTA, because of this difference in binding strength.

It is also worth noting that this differential in binding strength is not confined to iron (Table III). DTPA has a greater affinity for strontium and cobalt than does EDTA,[18] and might therefore be more effective in removing

these metals from the human body. Schubert and Fried[19] have shown that up to 35 per cent of an intravenous dose of monomeric thorium 234 may be recovered from animals by chelation with DTPA. Winchell and co-workers,[20] using yttrium 90–DTPA to administer total body irradiation to animals, found that the chelated Y^{90} was rapidly excreted after intravenous injection. DTPA may thus prove useful in the removal of accidentally acquired radioisotopes, a constantly increasing industrial (not to mention military) hazard.

The clinical application of DTPA in hemochromatosis is, of course, not yet defined, and further studies are in progress to determine long-term efficiency. Although intravenous administration is cumbersome, the intramuscular route offers obvious advantages. Our data indicate that absorption from the intramuscular site is complete, in that iron excretion was equivalent when the same dose of DTPA was given intramuscularly and intravenously. The drug may offer some promise in those cases of iron storage disease where phlebotomy is contraindicated and may also be used to supplement phlebotomy and thus reduce the treatment period necessary. In the average case of hemochromatosis, 2 to 3 years of weekly phlebotomy is required to completely eliminate the excess iron.

SUMMARY

The results of parenteral administration of the chelate trisodium calcium diethylenetriaminepentaacetate (DTPA) in five patients with iron storage disease are reported. DTPA given intravenously in doses of 2.5 to 4.0 Gm. resulted in iron excretion of up to 109 mg. per 24 hours in three patients with clinical hemochromatosis. Two control subjects showed no increase in urinary iron after the chelate was given.

There was little, if any, clinical toxicity, and no significant effect on hemogram, urinalyses, blood urea nitrogen, or serum calcium. Prothrombin times were only slightly prolonged. Comparable iron excretion occurred after intravenous and intramuscular administration of DTPA.

The amount of iron excreted in various subjects correlated best with the estimated iron stores. The hypothesis is advanced that parenterally administered DTPA is carried in the circulation to iron storage sites, the calcium in the chelate is exchanged for iron, and this complex continues in the circulation and is excreted in the urine.

The increased efficiency of DTPA over EDTA is discussed. The clinical application of DTPA is not established but the results reported here suggest a possible role as a supplement to phlebotomy in the treatment of hemochromatosis and as an alternative therapy for individuals with iron storage disease who could not undergo phlebotomy.

REFERENCES

1. Balfour, W. M., Hahn, P. F., Bale, W. F., Pommerenke, W. T., and Whipple, C. H.: Radioactive Iron Absorption in Clinical Conditions; Normal, Pregnancy, Anemia, and Hemochromatosis, J. Exper. Med. **76**: 15, 1942.
2. Finch, C. A.: Iron Metabolism in Hemochromatosis, J. Clin. Invest. **28**: 780, 1949.
3. Finch, S. C., and Finch, C. A.: Idiopathic Hemochromatosis, an Iron Storage Disease, Medicine **34**: 381, 1955.

4. Foreman, H., Huff, R., Oda, J., and Garcia, J.: Use of a Chelating Agent for Accelerating Excretion of Radio-iron, Proc. Soc. Exper. Biol. & Med. **79**: 520, 1952.
5. Ohlsson, W. T., Kullendorff, G. T., and Wungberg, K. L.: Transfusion Hemosiderosis; Report of a Case Treated With BAL, Acta med. scandinav. **145**: 410, 1953.
6. Wishinsky, H., Weinberg, T., Prévost, E. M., Burgin, B., and Miller, M. J.: Ethylenediaminetetraacetic Acid in the Mobilization and Removal of Iron in a Case of Hemochromatosis, J. LAB. & CLIN. MED. **42**: 550, 1953.
7. Seven, M. J., Gottleib, H., Israel, H. L., Reinhold, J. G., and Rubin, M.: N-Hydroxyethylene Diamine Triacetic Acid Versenol in the Treatment of Hemochromatosis, Am. J. M. Sc. **228**: 646, 1954.
8. Greenwalt, T., and Ayers, V. E.: Calcium Disodium EDTA in Transfusion Hemosiderosis, Am. J. Clin. Path. **25**: 266, 1955.
9. Figueroa, W. G., Adams, W. S., Bassett, S. H., Rosove, L., and Davis, F.: Effect of Disodium Calcium Versenate on Iron Excretion in Man, Am. J. Med. **17**: 101, 1954.
10. McMahon, F. G.: A Comparison of the Effects of Fe-3-Specific, Versenol, and Calcium Disodium Versenate on Urinary Iron excretion in a Patient With Hemochromatosis, J. LAB. & CLIN. MED. **48**: 589, 1956.
11. Figueroa, W. G., Adams, W. S., Davis, F. W., and Bassett, S. H.: A Study of the Effect of Disodium Calcium Versenate (Ca EDTA) on Iron Excretion in Man, J. LAB. & CLIN. MED. **46**: 534, 1955.
12. Rubin, M., Houlihan, J., and Princiotto, J. V.: Chelation and Iron Metabolism. I. Relative Iron Binding of Chelating Agents and Siderophilin in Serum, Proc. Soc. Exper. Biol. & Med. **103**: 663, 1960.
13. Rubin, M., and Princiotto, J. V.: Synthetic Amino Acid Chelating Agents and Iron Metabolism, Ann. New York Acad. Sc. In press.
14. Ramsay, W. N. M.: The Determination of Iron in Blood Plasma or Serum, Biochem. J. **53**: 227, 1953.
14a. Ramsay, W. N. M.: An Improved Technique for the Determination of Plasma Iron, Biochem. J. **57**: xvii, 1954.
15. Rath, C. E., and Finch, C. A.: Chemical, Clinical and Immunological Studies on the Products of Human Plasma Fractionation. XXXVIII. Serum Iron Transport. Measurement of Iron-Binding Capacity of Serum in Man, J. Clin. Invest. **28**: 79, 1949.
16. Rath, C. E., and Finch, C. A.: Sternal Marrow Hemosiderin, J. LAB. & CLIN. MED. **33**: 81, 1948.
17. Beutler, E.: Iron Enzymes in Iron Deficiency. I. Cytochrome C, Am. J. M. Sc. **234**: 517, 1957.
18. Chaberek, S., and Martell, A. E.: Organic Sequestering Agents, New York, 1959, John Wiley & Sons, Inc., pp. 572, 580.
19. Schubert, J., and Fried, J.: Chelating Agents in the Treatment of Poisoning by Polymerizable Radioelements, Nature, London, **185**: 551, 1960.
20. Winchell, H. S., Pollycove, M., and Lawrence, J. H.: Total Body Irradiation Using DTPA Chelated Y^{90}, Clin. Res. **8**: 220, 1960.

THE BINDING AND TRANSPORT OF IRON BY TRANSFERRIN VARIANTS

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Eight genetically controlled variations in human transferrin have been found by starch gel electrophoresis. An investigation was undertaken to determine whether the structural differences in these proteins are associated with any alteration in their ability to transport iron.

The binding of radioiron by different transferrins was studied in vitro using electrophoresis on paper and in starch gel. In experiments of short duration, in which sera that contained different transferrins were mixed, there was little or no transfer of iron from one unsaturated transferrin to another. Transferrins C and D_2 did not differ in their avidity for added iron or in their rate of iron binding.

The transfer of iron from four different transferrins to the tissues was investigated in vivo by simultaneous measurement of plasma clearance and subsequent utilization of one radioisotope of iron bound to plasma that contained the common transferrin (C) and the other to plasma that contained an unusual transferrin. Similar clearance and utilization of the two isotopes indicated similar rates of transfer of iron from the different transferrins to the tissues.

These studies indicate that those transferrins which have been investigated do not differ essentially in their ability to transport iron and deliver it to the tissues for utilization or storage.

GENETICALLY controlled variations in human transferrin (siderophilin), the iron-binding plasma protein, were first demonstrated by Smithies[1] in 1957. Subsequently it was found[2-5] that there are at least eight transferrins which are differentiated by their varying electrophoretic mobility in starch gel. They have been named in order of decreasing mobility transferrin B_0, B_1, B_2, C, D_0, D_1, D_2, and D_3. Fig. 1 is a diagram of the electrophoretic patterns which have been observed. This polymorphism is thought to represent a series of allelomorphic genes at a single locus. Most individuals have the single transferrin C and are presumed to be homozygous for the gene Tf^c. About 1 per cent of apparently healthy white persons have both transferrin C and the faster moving B_2; their transferrin phenotype is thus B_2C. The phenotype CD_1 is found in about 10 per cent of American Negroes and is even more prevalent among Australian aborigines. The remaining types are apparently very rare.

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The present study was undertaken to determine whether the variations in the molecular structure of the transferrins, indicated by the differing electrophoretic mobility, are associated with any measurable differences in the binding and transport of iron.

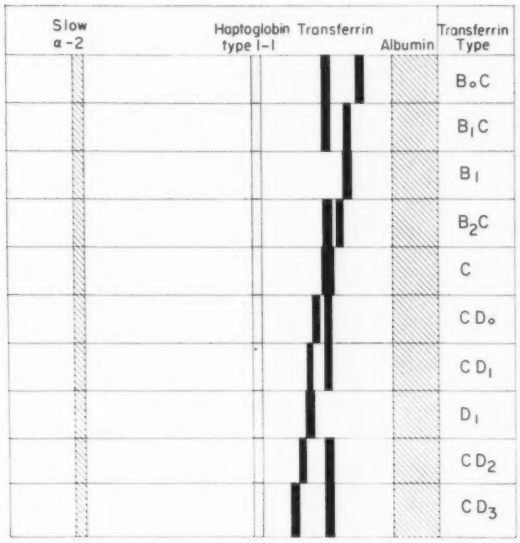

Fig. 1.—Diagram of appearance on electrophoresis in starch gel of the known transferrin variants in relation to haptoglobin (type 1-1) and albumin.

MATERIALS AND METHODS

In Vitro Experiments.—The amount and rate of iron binding and the transfer of iron from one transferrin to another were studied in vitro. Appropriate amounts of Fe^{59}* (as ferric chloride diluted with saline) were added to aliquots of unbuffered serum which were then subjected to electrophoresis at pH 8.6 either in starch gel by the method of Smithies[6] or on paper strip using a Durrum cell (Spinco type R) and 0.075 ionic strength Veronal buffer. Autoradiographs of starch gels were made as previously described.[5]

As is shown in Fig. 2 the fastest and slowest migrating transferrins, B_0 and D_3, can be differentiated from transferrin C by electrophoresis on paper. In CD_3 serum the separation is sufficient to allow more accurate quantitation of radioiron bound to the two proteins than can easily be achieved using starch gel. Most of the in vitro studies were therefore made on CD_2 serum.

Localization of the transferrin bands by staining proved unsatisfactory since iron is eluted from the protein at the acid pH required in the usual staining procedures. Accordingly, the unstained strips were firmly fixed to x-ray film and a pattern of pinholes was made through the film and paper before autoradiography. The radioactive bands were identified by tracing the areas of darkened film on the paper after exact alignment of the pinholes in front of a lighted screen. These areas were cut from the strips and counted in a well-type scintillation counter.

In Vivo Studies.—The transfer of iron from the transferrins to the tissues was studied by a double isotope technique using Fe^{59} and Fe^{55}.† The simultaneous disappearance from the plasma and subsequent utilization of radioiron bound in vitro to two different transferrins was measured by the method of Bothwell and collaborators.[7] Individuals of transferrin type

*Specific activity 20 μc per microgram.
†Specific activity 3.8 μc per microgram.

C were given by intravenous injection one isotope bound to 10 ml. of their own plasma and the other bound to 10 ml. of plasma from a donor of unusual transferrin type. Each subject received about 4 μc Fe^{59} and 30 μc Fe^{55}. In view of the difference in specific activity, carrier iron (Fe^{56}) was added to the Fe^{59} so that equal amounts of iron (as ferric citrate) were added to the two plasmas without exceeding the iron-binding capacity. Measurements were made of the iron content and iron-binding capacity of the labeled plasmas which were

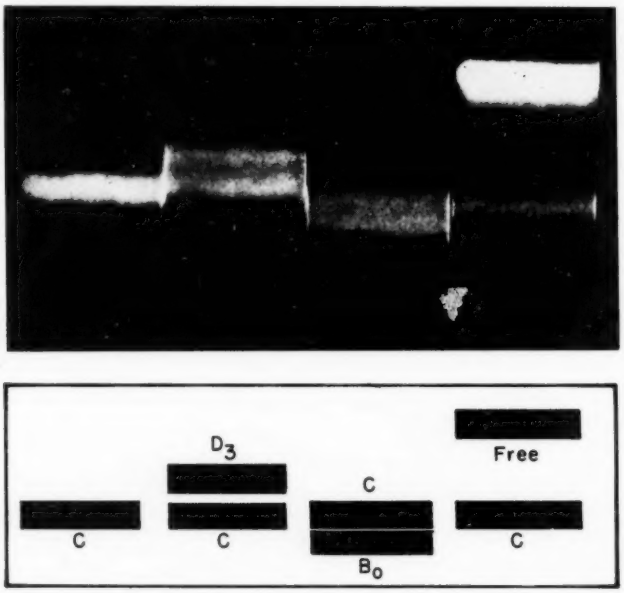

Fig. 2.—Autoradiograph of paper strips after electrophoresis at pH 8.6 of sera labeled with Fe^{59}. Left to right: transferrin type C, type CD_3, type B_oC, and type C oversaturated with iron to show unbound (free) iron at point of application.

TABLE I

RECIPIENT	HEMOGLOBIN (GM./100 ML.)	PLASMA IRON (μG./100 ML.)	TOTAL IRON-BINDING CAPACITY (μG./100 ML.)	LABELED PLASMAS: RECIPIENT: TRANSFERRIN TYPE	RECIPIENT: ISOTOPE	RECIPIENT: % SATURATION OF TRANSFERRIN	DONOR: TRANSFERRIN TYPE	DONOR: ISOTOPE	DONOR: % SATURATION OF TRANSFERRIN	T½ (MIN.) OF DECLINE IN PLASMA RADIOACTIVITY: Fe^{55}	T½ (MIN.) OF DECLINE IN PLASMA RADIOACTIVITY: Fe^{59}	PERCENTAGE OF ISOTOPE IN BLOOD AT 14 DAYS: Fe^{55}	PERCENTAGE OF ISOTOPE IN BLOOD AT 14 DAYS: Fe^{59}
B. B.	14.6	98	309	C	Fe^{55}	60	C	Fe^{59}	68	65	67	85	84
G. J.	15.9	147	309	C	Fe^{55}	87	C	Fe^{59}	68	93	97	86	82
A. V.	13.5	109	260	C	Fe^{55}	70	C	Fe^{59}	68	101	103	87	91

Plasma disappearance rates and percentage utilization for hemoglobin synthesis in normal subjects after intravenous injection of Fe^{55} bound to recipient's own plasma and Fe^{59} bound to donor plasma of the same transferrin type (C).

mixed immediately before injection. The weight of the injected plasma and of an aliquot set aside as a standard was accurately determined. At least four blood samples were drawn at suitable intervals for a 2 hour period and the plasma was separated by centrifugation. A further blood sample was obtained at 14 days. To provide a check on the subsequent processing, the γ emission of Fe^{59} was measured with a well-type scintillation counter on aliquots of all samples and suitable dilutions of the standards. The samples and standards

were then digested, and the iron was electroplated onto copper disks for differential Geiger-Müller counting by the method of Peacock and co-workers.[8] The standards and whole blood samples were processed in triplicate.

The accuracy of these measurements was assessed in three healthy subjects who were given Fe^{55} bound to their own plasma and Fe^{59} bound to plasma from a donor of the same transferrin type (C). The results of these observations are shown in Table I.

Hemoglobin was measured as oxyhemoglobin in an Evelyn colorimeter at 540 mμ. Hematocrits were spun in Wintrobe tubes at 2,750 g for 30 minutes. Plasma iron was measured by the method of Bothwell and Mallett,[9] and iron-binding capacity was measured by the method of Ressler and Zak.[10] Blood volume was calculated from the initial dilution of the injected Fe^{59}, assuming a normal body hematocrit to venous hematocrit ratio of 0.92. Plasma iron turnover was calculated from the disappearance of the isotope bound to the subject's own transferrin and was expressed in milligrams per day per 100 ml. whole blood.[7]

RESULTS

In Vitro Experiments.—

Interchange of iron between two transferrins: Serum of transferrin type C was labeled with Fe^{59} (about 1.2 μg per milliliter of serum) and incubated for 30 minutes at 37° C. Equal volumes of this labeled type C serum and unlabeled serum of types B_0C and CD_3 were then mixed and incubated for 30 minutes.

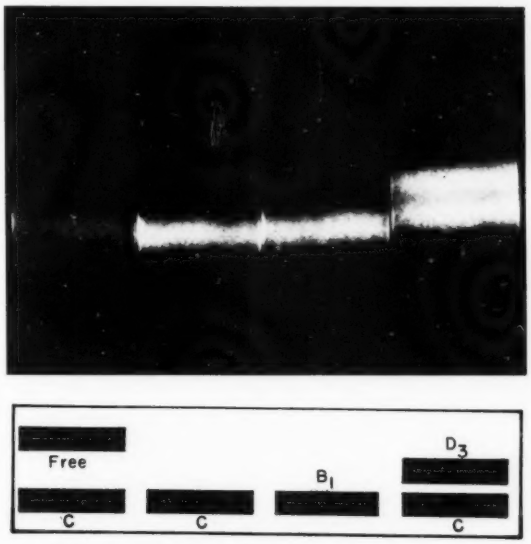

Fig. 3.—Autoradiograph of paper strips after electrophoresis at pH 8.6 of (left to right) labeled transferrin type C serum oversaturated with iron to show unbound iron at point of application, labeled C serum, unlabeled CD_3 serum mixed with labeled B_1 serum, and labeled CD_3 serum. Note lack of radioactivity in D_3 band in unlabeled CD_3 serum mixed with labeled B_1 serum showing absence of transfer of radioiron from B_1 to D_3 (see text).

For comparison with these mixtures further samples of B_0C and CD_3 serum were directly labeled with Fe^{59}. After overnight storage at 4° C. and exposure to room temperature for 2 hours all specimens were subjected to electrophoresis in starch gel. An autoradiograph of the gel showed that, whereas there was radioactivity in both transferrin bands of the directly labeled CD_3 and B_0C sera, there was radioactivity only in the transferrin C band in the mixtures prepared

from labeled C serum and unlabeled CD_3 and B_0C sera. Thus, under these conditions there was no detectable transfer of radioiron from transferrin C to either transferrin B_0 or D_3.

In a similar experiment labeled B_1 serum was incubated for 30 minutes with unlabeled C serum and the mixture was subjected to electrophoresis in starch. An autoradiograph of the gel showed no sign of transfer of radioiron from transferrin B_1 to transferrin C.

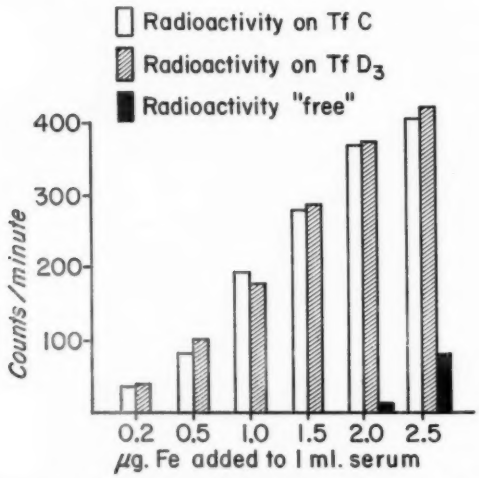

Fig. 4.—Radioactivity on transferrins C and D_3 after addition of increments of Fe^{59} to serum of transferrin type CD_3 from subject M. S.

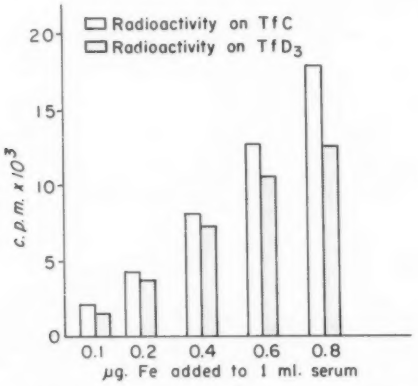

Fig. 5.—Radioactivity on transferrins C and D_3 after addition of increments of Fe^{59} to serum of transferrin type CD_3 from subject A. S.

In a third experiment labeled B_1 serum was incubated for 30 minutes with unlabeled CD_3 serum. After electrophoresis on paper no transfer of radioiron from the B_1 to the D_3 transferrin could be detected by autoradiography (Fig. 3). When the radioactivity in the protein bands was measured in the scintillation counter, the D_3 band was found to contain about 4 per cent of the added radioiron. This was probably due to incomplete separation of the B_1 and D_3 transferrins,* but it may indicate a small amount of iron transfer.

*B_1 and C transferrins are not separated by one dimensional electrophoresis on paper.

Amount of iron bound by two transferrins in same serum: Serum of type CD_3 was placed in a series of small tubes to which were added increments of Fe^{59} ranging from 0.2 to 2.5 μg per milliliter of serum. The radioactivity in the two transferrins was measured after electrophoresis on paper. As shown in Fig. 4, there was a proportional and equal rise in the radioactivity in the transferrins up to the level of saturation at which point unbound iron appeared at the origin.

These findings indicate that the two transferrins did not differ in avidity for iron and that, as measured by iron-binding capacity, they were present in the same quantity. By the same criterion sera from two near relatives also contained equal amounts of these two transferrins. In contrast, a fourth member of the family appeared to have a larger amount of unsaturated transferrin C (in terms of iron-binding capacity) than of transferrin D_3, since each addition of radioiron was associated with a proportionately greater increment of radioactivity on transferrin C (Fig. 5).

Rate of iron binding by two transferrins in same serum: One tenth microgram of Fe^{59} in 0.1 ml. saline was added to each of a series of tubes that contained 0.25 ml. of serum of type CD_3. At intervals of 0, 5, 12, 20, and 60 seconds after the addition of the radioiron its reaction with transferrin was virtually stopped by the rapid admixture of 1 μg of carrier iron (as ferric ammonium sulfate), which far exceeded the binding capacity of the serum. The amounts of radioactivity bound to the two transferrins after paper electrophoresis are shown in Fig. 6. In each tube the protein-bound radioactivity was equally divided between the two transferrins which thus appeared to bind iron at the same rate.

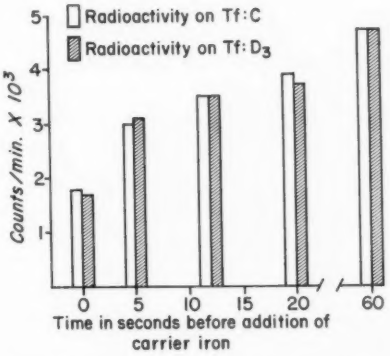

Fig. 6.—Radioactivity on transferrins C and D_3 after successive additions of Fe^{59} and carrier iron (Fe^{56}) to serum of transferrin type CD_3 from subject P. S.

In Vivo Observations.—The plasma clearance and effective utilization of iron bound to different transferrins was measured in six patients of transferrin type C. For Cases 1 and 2 the donor plasma was of transferrin type B_1. Plasma from heterozygous donors of transferrin type B_0C and CD_3 was used for Cases 3 to 6, B_0 having the fastest, and D_3 the slowest migration rate of the known transferrins. Table II summarizes the pertinent data for each of the patients studied. The plasma clearance of each isotope is given as the half-time

TABLE II

RECIPIENT		DIAGNOSIS	HEMOGLOBIN (GM./100 ML.)	PLASMA IRON (μG./100 ML.)	TOTAL IRON-BINDING CAPACITY (μG./100 ML.)	LABELED PLASMAS						T½ (MIN.) OF DECLINE IN PLASMA RADIOACTIVITY		PLASMA IRON TURNOVER (MG./DAY/100 ML. BLOOD)	PERCENTAGE OF ISOTOPE IN BLOOD AT 14 DAYS	
						RECIPIENT			DONOR							
						TRANSFERRIN TYPE	ISOTOPE	% SATURATION OF TRANSFERRIN	TRANSFERRIN TYPE	ISOTOPE	% SATURATION OF TRANSFERRIN	Fe^{55}	Fe^{59}		Fe^{55}	Fe^{59}
1.	G. M.	Pernicious anemia in therapeutic remission	13.9	89	245	C	Fe^{55}	69	B_1	Fe^{59}	96	81	80	0.59	84	83
2.	W. R.	Chronic lymphatic leukemia with hemolytic anemia	11.4	102	235	C	Fe^{55}	72	B_1	Fe^{59}	96	17	18	3.76	84	75
3.	E. K.	Myelomatosis	12.9	30	265	C	Fe^{55}	43	CD_3	Fe^{59}	73	32	31	0.56	93	91
4.	A. G.	Myelomatosis	11.3	45	396	C	Fe^{55}	35	CD_3	Fe^{59}	73	56	51	0.52	92	89
5.	B. S.	Chronic lymphatic leukemia	12.3	86	308	C	Fe^{59}	56	B_6C	Fe^{55}	80	82	78	0.67	86	87
6.	F. B.	Pernicious anemia in early relapse	12.6	100	282	C	Fe^{59}	65	B_6C	Fe^{55}	80	62	64	0.98	81	83

Plasma disappearance rates and per cent utilization for hemoglobin synthesis after intravenous injection in recipients of transferrin type C of one radioisotope of iron bound to the recipient's own plasma and the other bound to donor plasma of unusual transferrin type.

of the first exponential of radioactivity due to that isotope. Typical curves from two patients (Cases 1 and 2) are shown in Figs. 7 and 8. Plasma iron turnover was within normal limits in four cases. In agreement with the clinical data it was slightly increased in Case 6 and markedly so in Case 2, a patient with hemolytic anemia.

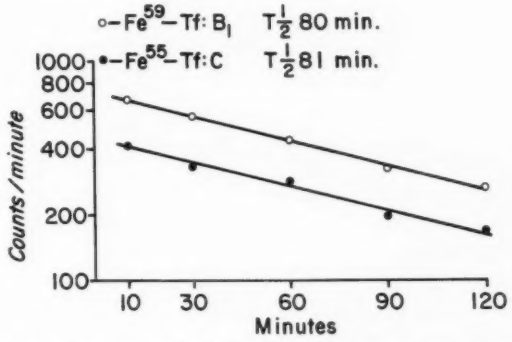

Fig. 7.—Plasma radioactivity in subject G. M. after intravenous injection of Fe^{55} bound to his own plasma (transferrin type C) and Fe^{59} bound to donor plasma of transferrin type B_1.

In spite of variations in the iron metabolism of the subjects the results show no consistent differences either in the plasma disappearance rates or in the percentage of the two isotopes utilized for hemoglobin synthesis. Such differences as were observed are not more than might be expected from the errors inherent in the measurements.

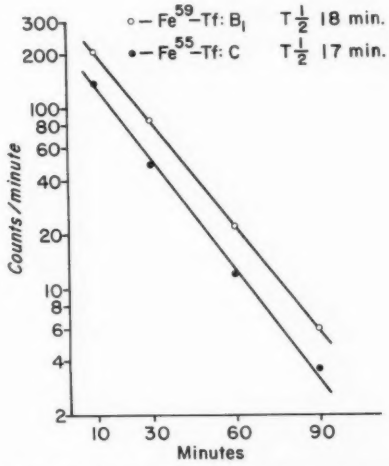

Fig. 8.—Plasma radioactivity in subject W. R. after intravenous injection of Fe^{55} bound to his own plasma (transferrin type C) and Fe^{59} bound to donor plasma of transferrin type B_1.

DISCUSSION

The occurrence of genetically controlled variations in transferrin suggests the possibility that these proteins differ in function as well as in electrophoretic mobility. Smithies[11] has pointed out that, since the variations in mobility are demonstrable both on paper and in starch gel, the transferrins probably differ

in electrical charge rather than in molecular size. Such differences might affect the role of transferrin in iron metabolism by altering either the binding of iron or its release to the tissues.

In vitro studies of the binding of iron by serum of type CD_3 suggest that these two transferrins, C and D_3, which by staining appear to be present in approximately the same quantity, bind iron at an equal rate and with equal avidity. The iron-binding capacity of these two proteins, and hence their amount in terms of this function, was the same in three of four sera tested.

The observation that different transferrins bind iron similarly in vitro does not necessarily mean that they function similarly in vivo, since they might differ in ability to release iron to the tissues. That they do not differ greatly in this respect is indicated by the in vivo studies in which the recipients received one isotope of iron bound to their own plasma and the other bound to plasma that contained one of the transferrin variants. Only small and inconstant differences were noted in the rates of disappearance from the plasma and in the proportions of the injected isotopes used for the synthesis of hemoglobin. These differences were of the same order as those seen in three healthy subjects who were given the second isotope bound to donor plasma of the same transferrin type as their own (C). The possibility was considered that these results might be affected by inequality in saturation of the two plasmas with iron, since this might alter the availability of the two isotopes. The data, however, show no correlation between the differences in saturation and the small differences in plasma clearance and utilization that were observed. On the assumption that there is as little exchange of iron between transferrins in vivo as was observed in vitro, it may be concluded that in each case the iron bound to the donor plasma was handled similarly to that bound to the subject's own transferrin. Thus, it appears probable that the various human transferrins do not differ essentially in their ability to bind iron and to transfer it to the tissues for utilization or storage.

Although the differences in the transferrins apparently do not appreciably affect the transport of iron, genetic considerations make it likely that they influence some function of this protein. Since few, if any, entirely neutral genes exist,[12, 13] the relatively high incidence of transferrin variants in certain populations in Africa and Australia suggests that these proteins are involved in some selective process. Mutation and genetic drift are unlikely to be entirely responsible for their persistence. If it is not the ability to transport iron, then some other property, such as the capacity to inhibit viral growth suggested by Martin and Jandl,[14] may be responsible for the perpetuation of the transferrin variants.

SUMMARY

The binding and transport of iron by the transferrin variants has been studied using the radioisotopes Fe^{59} and Fe^{55}.

In experiments of short duration in vitro there was little or no transfer of radioiron from one unsaturated transferrin to another.

Transferrins C and D_3 did not differ in their avidity for added iron or in their rate of iron-binding.

No consistent differences were observed in vivo in the plasma clearance or in the utilization of radioiron bound to four different transferrins.

It is concluded that the transferrin variants do not differ essentially in their ability to transport iron and deliver it to the tissues.

REFERENCES

1. Smithies, O.: Variations in Human Serum β-Globulins, Nature, London **180:** 1482, 1957.
2. Horsfall, W. R., and Smithies, O.: Genetic Control of Some Human Serum β-Globulins, Science **128:** 35, 1958.
3. Harris, H., Robson, E. B., and Siniscalco, M.: β-Globulin Variants in Man, Nature, London **182:** 452, 1958.
4. Smithies, O., and Hiller, O.: The Genetic Control of Transferrins in Humans, Biochem. J. **72:** 121, 1959.
5. Giblett, E. R., Hickman, C. G., and Smithies, O.: Serum Transferrins, Nature, London **183:** 1589, 1959.
6. Smithies, O.: An Improved Procedure for Starch-gel Electrophoresis: Further Variations in the Serum Proteins of Normal Individuals, Biochem. J. **71:** 585, 1959.
7. Bothwell, T. H., Hurtado, A. V., Donohue, D. M., and Finch, C. A.: Erythrokinetics. IV. The Plasma Iron Turnover as a Measure of Erythropoiesis, Blood **12:** 409, 1957.
8. Peacock, W. C., Evans, R. D., Irvine, J. W., Jr., Good, W. M., Kip, A. F., Weiss, S., and Gibson, J. G., II: The Use of Two Radioactive Isotopes of Iron in Tracer Studies of Erythrocytes, J. Clin. Invest. **25:** 605, 1946.
9. Bothwell, T. H., and Mallett, B.: The Determination of Iron in Plasma or Serum, Biochem. J. **59:** 599, 1955.
10. Ressler, N., and Zak, B.: Serum Unsaturated Iron-Binding Capacity, Am. J. Clin. Path. **30:** 87, 1958.
11. Smithies, O.: Zone Electrophoresis in Starch Gels and Its Application to Studies of Serum Proteins, *in* Anfinsen, C. B., Jr., Anson, M. L., Bailey, K., and Edsall, J. T., editors: Advances in Protein Chemistry, New York and London, 1959, Academic Press, Inc., vol. 14, pp. 65-113.
12. Fisher, R. A.: The Genetical Theory of Natural Selection, Oxford, 1930, Clarendon Press.
13. Ford, E. B.: Polymorphism, Biol. Rev. **20:** 73, 1945.
14. Martin, C. M., and Jandl, J. H.: Inhibition of Virus Multiplication by Transferrin, J. Clin. Invest. **38:** 1024, 1959.

LABORATORY METHODS

STUDIES WITH DOUBLY LABELED IRON

II. Separation of Iron From Blood Samples and Preparation of Ferrous Perchlorate for Liquid Scintillation Counting

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Chicago, Ill.

A rapid method for the preparation of samples containing Fe^{55} and Fe^{59} for ferrous perchlorate liquid scintillation counting is described. Organic materials—blood specimens—are digested and iron is crudely separated by precipitation. Iron is quantitatively isolated from digestates or certain inorganic solutions with a simple apparatus that permits rapid electrolytic separation. The plated iron is subsequently dissolved in dilute perchloric acid and after evaporation is ready for liquid scintillation counting.

A METHOD for the simultaneous counting of the isotopes Fe^{55} and Fe^{59} as ferrous perchlorate has been described in a previous article.[1] A procedure for the separation of iron from wet ashed blood samples by rapid electrodeposition and the preparation of ferrous perchlorate for liquid scintillation counting will be described. The conventional method for electroplating of iron from an oxalate solution has been modified for use with a simple apparatus which permits rapid quantitative electrodeposition of iron in a form which is readily reactive with dilute perchloric acid.

MATERIALS

Reagents.—All chemicals used are of reagent grade. They are: (1) sulfuric acid, concentrated, (2) perchloric acid, concentrated, 70 per cent, (3) perchloric acid, 0.4 M, (4) ammonium hydroxide, concentrated, (5) sodium sulfide solution, saturated (prepared fresh each week for iron test), (6) oxalic acid, saturated solution (at room temperature), (7) ammonium oxalate, saturated solution (at room temperature), (8) isotopes, obtained in the form of a sterile solution of ferrous sulfate, pH 4, and (9) ferric chloride solution (10 mg. iron per milliliter in 2N hydrochloric acid).

General Apparatus.—

1. Syringe type pipettes of appropriate size are used for all reagent deliveries except for concentrated acids and alkali. A 20 gauge needle is used as an orifice when precise delivery is indicated. Thorough rinsing, without disassembling the syringe, at the end of the run provides adequate cleaning.

2. Three hundred milliliter long neck Kjeldahl flasks.

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3. Centrifuge tubes, Pyrex, 90 ml. round bottom, heavy duty.

4. Counting vials, 20 ml. low activity glass with polyethylene cap.*

5. Water bath for the evaporation procedure consists of a shallow pan of approximately 5 cm. depth, with heat supplied by a heavy duty hot plate. Evaporation is carried out in the counting vials, and a suitable support can be made from sheet lead cylinders of approximately 5 cm. height and 32 mm. diameter, arranged in rows in the pan. Water between 95° and 100° C. should cover at least one half of the vial during the evaporation.

Electroplating Apparatus.—The electroplating device used in this laboratory is illustrated in Fig. 1, *A*. The electrode holders are constructed of 18 cm. lengths of 4 mm. diameter soft glass tubing into which is sealed† an 8 cm. length of 20 gauge platinum wire. Both anode and cathode holders are supported in a single thermometer clamp. The double anode (Fig. 1, *B*) is constructed of a length of 24 gauge platinum wire welded‡ at midpoint to the 20 gauge wire. Each arm of the anode terminates in a seven turn helix of approximately 3 mm. diameter. The arms of the anode are sprung outward slightly so that the helices will press firmly against the side walls of the tube when in position. Details of construction of the cathode are shown in Fig. 1, *C*. A simple connector for the detachable cathode is made by slipping a 1 cm. length of No. 18 Teflon§ tubing over the 20 gauge

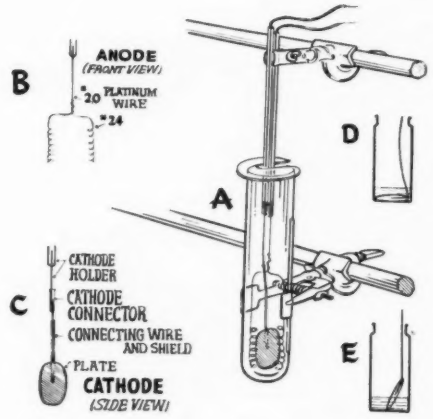

Fig. 1.—Apparatus for rapid electrodeposition of iron and preparation of ferrous perchlorate.

platinum wire of the cathode holder, and securing it in place by a small bend in the end of the platinum wire. Teflon, or an equivalent, should be used because of the high temperature developed during the electroplating process. The cathode proper is constructed of medium or heavy weight platinum foil (0.0015 to 0.002 inch) to which is welded a length of 24 gauge platinum wire. The cathode is secured by forcibly inserting the connecting wire of the cathode into the coupler on the cathode holder by means of a forceps. When in use, the cathode connecting wire is covered with a 3 cm. length of P.E. 60 polyethylene tubing¶ as a shield to prevent deposition of the iron on the connecting wire. The volume of sample lost in the space between the connecting wire and the tubing is negligible. The shield is discarded after a single use since cleaning is impractical.

In the assembled unit, the electrolyte is contained in a 90 ml. centrifuge tube supported in a burette clamp. The jaws of the clamp should be covered with asbestos rather than rubber sleeving. If necessary, a small wooden applicator stick can be inserted between the clamp and the tube to provide adequate friction to hold the tube in position. Slight

*Wheaton Glass Co., Millville, N. J.

†With soft sealing glass, Corning, for platinum to soft glass.

‡Welding of platinum is accomplished by holding the two pieces to be joined in the oxidizing portion of a Bunsen flame until near white heat. While still in the flame, the two pieces are firmly compressed with fine nose pliers.

§Erie Teflon Spaghetti No. 18, natural.

¶Clay-Adams Co., New York, N. Y.

loosening of the clamp will permit the tube to be moved smoothly downward with enough restriction of lateral motion to prevent contact between anode and cathode. A small disk of polyethylene sheet rests on top of the tube to prevent loss by spray. Electrical connection is made by mercury contact and copper wire. A number of similar cells can be mounted on a lattice easily constructed of conventional laboratory supports, clamps, and rods. Any direct current source capable of providing a potential of 10 volts and 3 amperes per cell is adequate. In this laboratory, a P.R. Mallory rectifier No. 12RS6DF is used. With this rectifier, a maximum of four cells can be operated simultaneously. The vigorous liberation of gas from the electrodes provides adequate stirring of the electrolyte. Sixteen samples per hour can be processed with the use of two sets of four electroplating cells, four samples being plated while the other four are prepared.

The detachable cathode is placed in the vial for the elution of the iron as shown in Fig. 1, *D*. Since platinum is somewhat brittle, it is recommended that the bend in the connecting wire be as smooth as possible to prolong the life of the electrode. Cathodes are cleaned by rinsing with alcohol and by washing with hot soap and water. Scrubbing of the plates with the fingers is necessary to remove a nonferrous, insoluble black film left on the plates after use. After immersion in 50 per cent hydrochloric acid and rinsing with water, the plates are flamed in the oxidizing portion of a Bunsen flame to remove any volatile matter. The polyethylene tubing is placed over the connecting wire and the electrodes are stored in a covered beaker.

Preparation of Samples, Standards, and Blanks.—The procedure was established for samples containing 10 mg. of iron. Although it has been shown that the method is valid for samples containing from 3 to 12 mg. of iron,[1] it is recommended that samples be adjusted to approximately 10 mg. of iron by the addition of an appropriate volume of ferric chloride solution.

Samples consist of heparinized blood. The volume of whole blood that contains 10 mg. of iron can be estimated: milliliters whole blood equals 10/hematocrit, or 100/hemoglobin (grams per cent) times 0.32.

Standards are usually prepared from the isotope containing material administered to the subject. If organic matter is present, a measured amount of the material should be digested by a process similar to that outlined except that reduced volume of reagents are used. A working standard is prepared from an aliquot of the material diluted (with 2N HCl) to reduce the activity to a reasonable counting level. Suitable aliquots of the working standard, each made to contain approximately 10 mg. of iron, are processed beginning at step 6 of the procedure. Either separate standards should be made for each isotope or a special Fe^{59} standard for the determination of cross counting can be prepared by the addition of 0.02 μc of Fe^{59} to 1 ml. of ferric chloride solution.

Procedure.—All volumes given are approximations except where specified.

A. *Digestion* (modified from Finch[2]):

1. Deliver an accurately measured volume of blood sample (containing approximately 10 mg. of iron) into Kjeldahl flask.
2. Add a glass bead and a volume of concentrated sulfuric acid equal to the volume of blood plus an 8 ml. excess.
3. Heat vigorously in perchloric acid fume hood until contents are oily and fuming (*no* solids left). (Approximately 1.5 hours or less.)
4. Cool and add 3 ml. of 70 per cent perchloric acid.
5. Heat (approximately 10 minutes) until contents are pale yellow in color but become colorless when cool. If not, add 1 ml. of 70 per cent perchloric acid and repeat step.

B. *Separation of iron:*

6. Transfer quantitatively into 90 ml. centrifuge tube.
7. Precipitate iron slowly with ammonium hydroxide and allow to settle.
8. Centrifuge; decant and discard supernatant.

C. *Electrodeposition:*

9. Add 1 ml. of oxalic acid solution and warm to dissolve precipitate.

10. Add 10 ml. of ammonium oxalate solution.

11. Place tube on electroplating apparatus and begin plating at 3 amperes per cell.

12. Add 1 ml. oxalic acid at 2, 4, 6, and 8 minutes.

13. At 12 minutes, slowly lower tube. Wash cathode with jet of distilled water during entire removal process.

14. Do not disconnect current until cathode is out of solution, then blot tip of cathode with tissue.

D. *Preparation of ferrous perchlorate:*

15. Measure exactly 2.0 ml. of dilute perchloric acid into counting vial, cover with watch glass, and place in water bath to heat.

16. Remove cathode from connector, discard polyethylene tubing shield, bend wire as shown in Fig. 1, *D*, and drop into counting vial immediately replacing watch glass.

17. Make certain that electrode is completely under solution. Heat for 5 minutes. (After the iron is dissolved, the procedure may be interrupted for 18 hours by capping and refrigerating vials.)

18. Remove vial cover, straighten the electrode wire (Fig. 1, *E*), and evaporate for 40 minutes.

19. Add counting reagents, remove electrode, and count as described previously.[1]

Notes on Procedure.—

Separation of Iron: The contents of the Kjeldahl flask can usually be transferred to the 90 ml. centrifuge tube with water, but if the digestion process has been unduly prolonged, a white adherent crust may remain in the flask. This can be loosened by adding a small amount of concentrated ammonium hydroxide which will turn the encrusted material brown. This will dissolve easily in a small amount of hydrochloric acid. Precipitation of iron is accomplished by the careful addition of concentrated ammonium hydroxide, with continuous vigorous stirring of the sample, until a gray precipitate forms (pH 4 to 5). After some settling has occurred, additional ammonium hydroxide is added until the supernatant reaches a pH of 7 to 8. The slow precipitation procedure usually eliminates problems of precipitate floating and peptization. Standards can be precipitated rapidly by the successive addition of a few drops of concentrated ammonium hydroxide and 50 ml. of distilled water and may be centrifuged immediately.

Electrodeposition of Iron: Although the crude precipitate dissolves easily in 1 ml. of warm saturated oxalic acid, some blood samples will remain cloudy. This will not interfere with the electrodeposition of iron.

In order to achieve quantitative electroplating of iron and to produce a deposit that is rapidly and completely soluble in 0.4M perchloric acid, the following conditions were found essential: (1) a current flow of 3 amperes per cell for the outlined electrolyte composition and volume, (2) avoidance of prolonged electrolysis, and (3) the use of a double anode system. With a current flow of 1.5 amperes, plating from blood digestates was quantitatively complete in 18 minutes. However, after immersion of the plate in 0.4M perchloric acid, a black residue (containing both iron and carbon) appeared, with a resulting counting loss of from 5 to 30 per cent. Less constantly, similar phenomena were observed with a current of 3 amperes if electroplating was continued for more than 15 minutes. The formation of an insoluble material occurred only rarely with deposition from a standard under conditions of low current density and prolonged plating even when phosphate, sulfate, and chloride were added to the standard in an attempt to simulate a blood digestate. When a single anode system was used, the insoluble residue invariably appeared with current flows of 3 amperes.

With this apparatus and electrolyte concentrations used, a current flow of 3 amperes will require an impressed potential of approximately 8 volts for blood digestates and 10 volts for standards. Plating both standards and blood digestates in parallel with the voltage adjusted to produce a mean current flow of 3 amperes per cell (approximately 2.7 amperes

for blood) has been found satisfactory. Current regulation is simplified by heating the electrolyte to near the boiling point and then adjusting the voltage to produce a current flow of 3 amperes per cell. During the plating process, the current flow may fall to as low as 2 amperes per cell but it has not been found necessary to correct this. If the electrolyte is not preheated, the voltage must be adjusted during the first two minutes of plating because of increasing conductivity as the electrolyte temperature rises.

Frequent additions of oxalic acid prevent the electrolyte from becoming too alkaline, although during most of the electrodeposition the solution is at an approximate level of pH 8. It has been possible to carry out a plating with only two additions of oxalic acid, but with a small safety factor. If the solution becomes too alkaline (brown discoloration), or too acid (failure to become water white after 8 to 10 minutes of plating), redigestion of the sample will be necessary, since precipitation of iron hydroxide quantitatively from an oxalate solution is difficult. This type of procedural accident is rarely seen under the conditions of plating outlined.

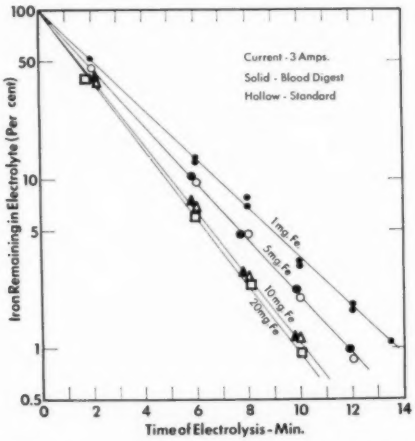

Fig. 2.—The rate of electrodeposition of iron from solutions containing from 1 to 20 mg. of iron. Abscissa, Duration of the electrolysis in minutes. Ordinate, Iron remaining in the electrolyte expressed as a percentage of the total amount present before electrolysis.

Studies of the rate of electrodeposition of iron from solutions that contain from 1 to 20 mg. of iron were carried out. Approximately 0.25 μc of Fe^{59} was added to a series of standards and blood digestates. During the electroplating procedure, 0.5 ml. aliquots were removed at 2 minute intervals for assay. Examples are presented in Fig. 2. The data indicate that the rate of electrodeposition is greater with progressively increasing amounts of iron in the electrolyte, and that the rate is essentially similar for deposition from blood digestates and standards. Although a 1 mg. sample plates fairly rapidly, the deposited iron was not always completely soluble in 0.4M perchloric acid.

It should be noted that with blood digestates, the plated iron appears as a dull, dark, gray film almost completely covering the cathode. With solutions in which the carrier iron was originally introduced as ferric chloride or perchlorate, plated iron appears as a barely visible, light gray, shiny deposit.

Preparation of ferrous perchlorate: Because of the quenching effect of perchlorate, it is desirable to keep the amount of perchloric acid used to dissolve the plated iron at a minimum. Very small volumes increase the chances for technical error, since it was found that the momentary emergence of the plate from the hot acid during elution resulted in the formation of difficultly soluble iron oxide and required redigestion. Current studies suggest the possibility of the use of 1.5 ml. of 0.32M perchloric acid.

An evaluation was made of the effect of the time of evaporation of the ferrous perchlorate solution on counting efficiency. Evaporation time was varied from 20 to 60 minutes and, in each case, the counting rates for the experimental samples were compared

to that observed with a sample evaporated for 40 minutes (reference sample). Data are presented in Fig. 3. A slight decrease in counting rate occurs with prolonged evaporations and it was noted that an occasional sample, which has been evaporated beyond the recommended time interval, may not become clear on the addition of the ascorbic acid reagent. In such cases, it is recommended that additional ascorbic acid be added in successive quantities of 1 to 2 mg. until decolorized.

After evaporation for 40 minutes, samples that contain 10 mg. of iron will appear as small amounts of moist green crystalline salt on the bottom of the vial. With less than 3 mg. of iron, the specimen will remain as a drop of viscous liquid. Little, if any, change in excess perchloric acid resulted from evaporation, as determined by titration to pH 3.5 with 0.01N NaOH. The ferrous perchlorate produced by this method appears to be the hexahydrate based on the weight of the residue and the amounts of ferrous perchlorate and perchloric acid present.

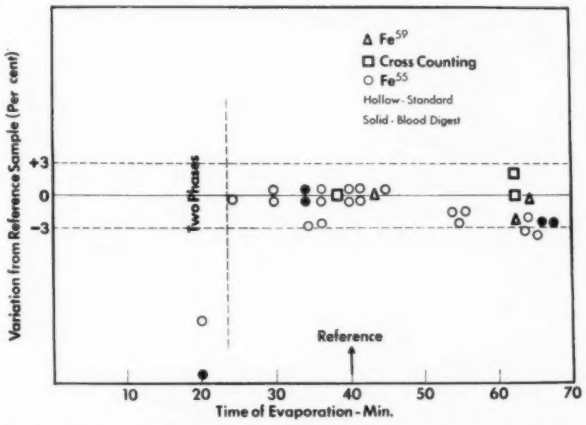

Fig. 3.—Effect of various lengths of time of evaporation of ferrous perchlorate solution on counting rate. Ordinate. The difference between the counting rates of an experimental and reference sample expressed as a percentage of that of the reference sample.

RESULTS

Reliability.—A series of 17 sample pairs (standard and blood digestate) was prepared for each isotope. Single, one minute counts were made. The mean percentile difference (and standard errors) between the counting rates of the standard and blood digestates were as follows: for Fe^{59}, + 1.12 ± 0.323 (S.E.), for Fe^{55}, − 0.0827 ± 0.198 (S.E.), for cross counting, − 0.435 ± 0.609 (S.E.). It is concluded from these data that the isotopes in blood samples can be assayed by comparison with standards and that cross counting, determined from standards, can be applied to blood samples.

In another study 7 groups were prepared, each containing duplicate Fe^{59} and Fe^{55} standards and one blood sample that contained both isotopes. Single, one minute counts were made for each specimen. In each group, the analysis of the sample was based on the standards of the particular group. The results are expressed as the mean percentile difference between the average counting rates of the duplicate standards and the sample: for Fe^{59}, − 0.897 ± 0.575 (S.E.) and for Fe^{55}, − 0.339 ± 0.299 (S.E.). It is concluded that the procedure offers analytic reliability.

In a recovery study, isotope was added to 2 blood samples which were processed as described and the counting rates were compared with those observed

when the same amount of isotope was added directly to the final counting mixture. Recovery was found to be 97.9 and 98.2 per cent for the two samples.

Interference by Other Tracers.—In order to study the degree of elimination of certain interfering tracers (P^{32} and Cr^{51}) by the electroplating procedure, the isotopes were added to nonradioactive blood samples and ferric chloride solutions, each containing 10 mg. of iron, as sodium phosphate and sodium chromate, respectively. With sodium chromate, almost complete coprecipitation with iron occurred both from blood digestates and ferric chloride solutions. During electroplating, from 1 to 3 per cent of the total Cr^{51} activity is deposited with iron on the cathode. At the photomultiplier voltage tap for counting Fe^{59} and Fe^{55}, Cr^{51} counts with efficiencies of the order of 1 and 6 per cent, respectively. It is evident that the isotope introduced by conventional Cr^{51} red cell tagging procedures can interfere with concomitant double iron isotope studies. With P^{32} added as sodium phosphate almost complete precipitation with alkali is again seen. When electroplating is carried out with iron standards free from additional carrier phosphate, a variable but significant amount (2 to 14 per cent) of P^{32} activity is deposited with the iron. With solutions that contain large amounts of phosphate such as blood, only 0.02 per cent of the activity was plated. In practice, P^{32} would be present in blood samples but not in standards. Therefore, in spite of P^{32} counting efficiencies of the order of 85 and 10 per cent of the voltage taps used for the determination of Fe^{59} and Fe^{55}, respectively, P^{32} tagging should present little interference if the amount of isotope is limited to tracer levels.

DISCUSSION

It is evident that the procedure offers technical simplicity. Precipitation of iron, formation of the oxalate, and electroplating are carried out in the same 90 ml. centrifuge tube. Preparation of ferrous perchlorate and counting of the specimen is carried out in the counting vial directly. The total time required from the beginning of digestion to the placing of the counting vial in the refrigeration unit is of the order of 3 to 3.5 hours.

Several other methods of preparation of ferrous perchlorate were considered. If the initial material is a simple salt of iron, it was found adequate to precipitate the iron as the hydroxide, wash, and dissolve in dilute perchloric acid. The direct precipitation of iron from a blood digestate carries most of the phosphate into the final mixture with the formation of a precipitate and a marked loss in counting efficiency. An analysis of the precipitate revealed a high phosphate content. Considerable experimentation with ion exchange resins indicated that satisfactory separation of iron could be accomplished but was less reliable and much more time consuming than the rapid electrodeposition process.

SUMMARY

1. A procedure is described for the separation of iron from blood samples and the preparation of ferrous perchlorate for simultaneous liquid scintillation counting of the isotopes Fe^{59} and Fe^{55}.

2. An apparatus for rapid quantitative electrodeposition of iron is described.

3. Interference by the tracers Cr^{51} and P^{32} on the determination of iron isotopes is evaluated.

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REFERENCES

1. Dern, R. J., and Hart, W. L.: Studies With Doubly Labeled Iron. I. Simultaneous Liquid Scintillation Counting Isotopes Fe^{55} and Fe^{59} as Ferrous Perchlorate, J. LAB. & CLIN. MED. **57**: 322, 1961.
2. Finch, C. A.: Personal communication.

A RAPID PROCEDURE FOR DETERMINATION OF MERCURY IN URINE AND KIDNEY

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A rapid and simple procedure for the determination of mercury in urine and kidney tissue is described. Potassium bromide is used to liberate mercuric ion from organic complexes and renders unnecessary any of the laborious manipulations usually employed in preparing samples for analysis. Free mercuric ion is then extracted in chloroform solutions of dithizone. The colored mercury dithizonate complex formed can be determined spectrophotometrically. Results using this method are equivalent to those obtained with a polarographic procedure. Renal and urinary mercury concentrations after administration of therapeutic doses to rats and dogs are comparable to those reported in the literature. The method may be used to study excretion of mercury in patients treated or poisoned with organic mercurial diuretics or inorganic mercury. As little as 10 mcg. mercury per milliliter of urine may be detected but, with minor changes, sensitivity can be adjusted for measurement of 1 mcg. mercury.

A NUMBER of procedures for the determination of mercury in biologic samples are available. All but the polarographic determination of urinary mercury involve extensive pretreatment of sample in order to convert organically bound mercury to inorganic mercuric ion, a laborious and time consuming process. A method which requires less attention to preparation of sample should be a useful tool both in the experimental and the clinical laboratory.

There are two distinct approaches by which such a method may be devised. The first is to eliminate the necessity of converting organic mercury to inorganic mercuric ion. In order to do this it is essential to demonstrate that the nature of the organic moiety and the group occupying the second valence of organic mercury do not influence the analytic reaction either qualitatively or quantitatively. The second approach is to simplify the manipulations necessary for the conversion of organically bound mercury to free mercuric ion.

This report describes analyses performed using the aforementioned approaches. Both were utilized successfully and provided rapid and simple means for determination of mercury in biologic samples.

METHODS

Standards.—Solutions of various mercurial compounds having the general structure, R-C-Hg^+ X^-, were prepared by dissolving known quantities in distilled water. In some cases

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0.1N NaOH was added and the pH was adjusted between 8.0 and 8.5 in order to dissolve a given compound. The mercurials used are listed in Table I. Group A includes labile compounds which can release free mercuric ion in acid solution. The 3 compounds in Group B are more stable and are resistant to fracture of the carbon-mercury bond.[1]

Unknowns.—Dog urine was collected through ureteral catheters before and after treatment with the mercurial compounds listed in Table I. Kidneys of control and mercurialized rats were removed, minced and homogenized with 6 to 8 parts of distilled water in test tubes using a motor driven rotary pestle. The homogenates were transferred to 50 ml. volumetric flasks. Approximately 30 ml. of 25 per cent trichloroacetic acid was added slowly with constant agitation of the flasks. The flasks were then shaken vigorously 200 times before adding 10 ml. of a 40 per cent solution of KBr. Volume was adjusted to 50 ml. with distilled water. One gram portions of dog kidney cortex were prepared in the same fashion.

Analytic Procedure.—Aliquots of standard solutions of urine in volumes of 0.1 to 1.0 ml. were pipetted into 120 ml. glass bottles fitted with plastic screw-caps. The following reagents were added in order with gentle mixing after each addition: 5 ml. 10 per cent trichloroacetic acid, 5 ml. 40 per cent KBr, 35 ml. of 0.5M formic acid buffer (pH 5.0). Potassium bromide was omitted in some of the samples. Exactly 10 ml. of chloroform solution of dithizone (20 mg. per liter) was then added from a burette and the bottles were shaken vigorously 200 times to extract mercury from the aqueous phase. The resultant mercury dithizonate in the chloroform phase was poured into test tubes after removal of the aqueous phase by suction. Since emulsions were never formed centrifugation was unnecessary. Transmission values were determined at 475 mμ using a Bausch & Lomb spectrophotometer (Spectronic "20").

TABLE I. ORGANIC MERCURIAL COMPOUNDS (GENERAL STRUCTURE: R-C-Hg^{+} X^{-})

COMPOUND	Hg^{++} RELEASED IN ACID
Group A	
Mercaptomerin	yes
Chlormerodrin	yes
Salyrganic acid	yes
Salyrgan (with theophylline)	yes
Meralluride	yes
p-OH Ø HgX	yes
$CH_3OCH_2CH_2HgX$	yes
Group B	
o-OH Ø Hg X	no
p-COOH Ø HgX	no
CH_3HgX	no

Five or 10 ml. aliquots of homogenate were taken for analysis and buffered with 50 ml. formate (pH 5.0) without further addition of acid or KBr.

Transmission of standard solutions was estimated with reagent blanks set at 100 per cent transmission. Control urine served as blank for urine mercury analysis. There was no significant difference between control urine and reagent blanks. Transmission of tissue homogenates prepared with kidney of untreated rats usually was 95 per cent or more of reagent blanks. No correction was applied in the case of dog renal cortex for which a reagent blank was employed.

All reagents used were analytic grade. Traces of heavy metal were removed from KBr solutions by shaking with dithizone as described by Laug and Nelson.[2]

RESULTS AND DISCUSSION

Standard curves relating extinction (E) of mercury dithizonate to mercury concentration are shown in Fig. 1. Curve A describes the relationship obtained when inorganic mercuric ion is reacted with dithizone with or without prior

addition of KBr to form the complex salt, K_2HgBr_4. All acid-labile organic mercurials ($R–Hg^+$) tested fall on the same curve when KBr is used. In the absence of bromide ion the same mercurials in equal concentration react with only one half the quantity of dithizone (curve *B*). Organic compounds of mercury which do not split off free mercuric ion in the presence of KBr also follow curve *B*. It was not possible to double the slope of the stable compounds (listed in Table I) by adding bromide to the reaction mixture. These results indicate that dissociable organic mercuric salts with a single free balance react with dithizone in a 1:1 molar ratio. Fracture of the carbon-mercury bond in such compounds splits off a free mercuric ion which reacts with two molecules of dithizone.

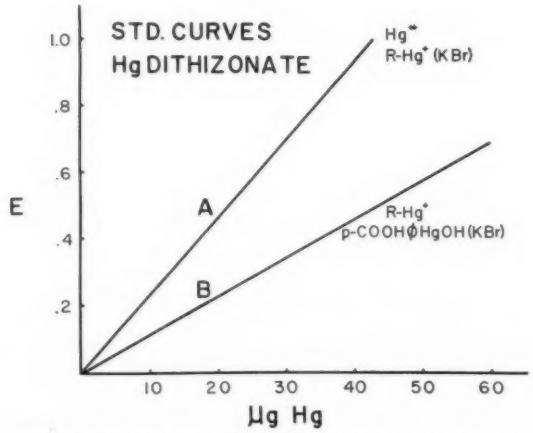

Fig. 1.—Standard curves relating extinction (*E*) to concentration of mercury. Curve obtained using a spectrophotometer. See text for details.

Similar results were obtained when organic or inorganic mercury was added in concentrations of 5 to 1,000 mcg. per milliliter of urine. Recovery was 100 per cent and not dependent on the use of bromide ion. Urinary mercury concentration in dogs treated with Salyrganic acid is shown in Table II. In these experiments the animals were anesthetized with pentobarbital and received 4 mg. mercury per kilogram intravenously. Urine was collected for 2 hours and analyzed for mercury by the technique described using bromide ion and by the polarographic method employed by Weiner and Müller.[3] The first urine sample obtained from Dog 3 contained blood and was discarded. Results presented in Table II show little difference between the 2 methods. When analyses were performed without addition of bromide, urinary concentrations were calculated using curve *B* (Fig. 1) and were found to be 20 to 40 per cent higher than those obtained by the polarographic procedure. This was probably due to breakdown of a fraction of the organic complex with subsequent excretion of free mercuric ion or actual fracture of the carbon-mercury bond in the urine during the collection period. For this reason it is more feasible to convert to mercuric ion by formation of K_2HgBr_4.

It was not possible to recover mercury added to kidney homogenates without addition of bromide ion. Recovery of mercury added to dog and rat kidney

homogenates incubated at 37° C. for 30 minutes ranged from 80 to 120 per cent with a mean value of 93 per cent when bromide was used. The amount of mercury present in kidney tissue was dependent on dose administered, time after administration, and the nature of the mercurial used. Concentration in dog renal cortex 2 hours after administration of 1 to 4 mg. mercury per kilogram ranged from 8 to 26 meg. per gram cortex for mersalyl and 120 to 130 meg. per gram cortex for chlormerodrin. These values are equivalent to those reported by Kessler et al.[4] using Hg^{203}-labeled compounds.

TABLE II. DITHIZONE-KBR METHOD VERSUS POLAROGRAPHIC METHOD FOR DETERMINATION OF URINARY MERCURY

		MERCURY CONCENTRATION IN DOG URINE (μG HG/ML.)		
ANIMAL	SAMPLE	DITHIZONE METHOD	POLAROGRAPHIC METHOD	% DIFFERENCE
1	First hour	182.4	174.7	+4.2
	Second hour	30.0	28.6	+4.7
2	First hour	166.0	168.3	-1.4
	Second hour	44.0	42.4	+3.7
3	Second hour	43.8	46.5	-6.2

Since mercury concentrations present in urine and kidney tissue when therapeutic doses are employed are extremely high, it is unnecessary to extract trace metals present in urine or renal tissue. The method can be used to detect quantities ranging from 5 to 50 meg. of mercury in 1 ml. of urine or 1 gram of tissue. Larger quantities can be determined readily by diluting the sample or by using smaller aliquots of sample. This will usually be necessary for estimating mercury excretion after administration of a therapeutic dose or in cases of acute mercury intoxication. Modification of the method is required for detection of 1 to 5 meg. mercury per milliliter of urine. Since emulsions are formed when urine volumes larger than 1 ml. are used, the best procedure is to increase the sensitivity of the colorimetric analysis by employing more dilute solutions of dithizone. Trace metals may be extracted from all reagents and samples by shaking with dithizone as described by Laug and Nelson.[2]

SUMMARY

A rapid and simple procedure for determination of mercury in urine of animals treated with therapeutic doses of organic mercurials is described. Trichloroacetic acid is used to remove traces of protein and also to lower the pH of urine so that organic mercury may complex with potassium bromide to form potassium mercuric bromide. Elevation of pH by addition of formate buffer (pH 5.0) permits the total extraction of mercury as the colored dithizonate.

The method can be applied to determination of tissue mercury.

I am indebted to Charles Ross and Z. Shaikh for technical assistance and to Dr. I. M. Weiner for supplying purified preparations of many of the mercurial compounds used in the investigation.

REFERENCES

1. Mudge, G. H., and Weiner, I. M.: The Mechanism of Action of Mercurial and Xanthine Diuretics, Ann. New York Acad. **71**: 344, 1958.
2. Laug, E. P., and Nelson, K. W.: Report on Mercury, J. A. Offic. Agr. Chemists **25**: 399, 1942.
3. Weiner, I. M., and Müller, O. H.: A Polarographic Study of Mersalyl (Salyrgan)—Thiol Complexes and of the Excreted Products of Mersalyl, J. Pharmacol. & Exper. Therap. **113**: 241, 1955.
4. Kessler, R. H., Lozano, R., and Pitts, R. F.: Studies on Structure Diuretic Activity Relationships of Organic Compounds of Mercury, J. Clin. Invest. **36**: 656, 1957.

A SERUM FORTIFICATION TECHNIQUE FOR THE MEASUREMENT OF ANTIBODY IN LOW TITER SERA

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This report describes an immunologic technique useful for detecting and measuring slight amounts of free antibody, particularly in serum of essentially low titer such as are frequently encountered in chronic disease states. The technique is essentially one in which dilutions of a known high titered antiserum to the homologous antigen is so mixed with equal samples of a serum of unknown homologous antibody strength that when the reactive strength of the mixture is determined and subtracted from the known strength of the fortifying serum, the difference can be assigned as the antibody strength of the unknown serum. This technique in flexible and accommodates variations to the extent that it might be applied to many different investigations. Illustrative experimental data derived through application of this technique to the demonstration of mycobacterial precipitins are included.

INVESTIGATIONS have been done to determine if a gel double diffusion precipitation technique was suitable for the detection and analysis of mycobacterial antibody.[1-5] The method, a modified Oakley gel double diffusion tube technique, was developed[5] and was found to be a reliable, sensitive method for detecting mycobacterial precipitins in mouse,* guinea pig,* monkey,* rabbit,[1, 2, 5] and human[3-5] sera when unheated, concentrated culture filtrate preparations were used as antigens.

All experience and data obtained from studies of tuberculous sera using the gel double diffusion reaction support the contention that detectable precipitin titers in sera from both animals and humans with active tuberculous disease are frequently very low. This observation has led to some further investigations directed toward the development of increasingly more sensitive techniques for the detection of mycobacterial antibody.

The major purpose of this report is to present a new immunologic method, *serum fortification.* To date, this technique has been applied exclusively to precipitating systems which are demonstrable by gel double diffusion methods; however, the general principles of serum fortification might be equally applicable to other immunologic systems. As an illustration of the usefulness of this new method, some experimental data obtained through application of serum fortification analysis for detection of mycobacterial precipitins in human sera are given.

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MATERIALS AND METHODS

In principle, serum fortification refers to a process by which a serum of low precipitating strength is fortified through the addition of measured, graded amounts of a second homologous antiserum known to have a relatively high precipitin titer (fortifying serum). This procedure is outlined.

1. *Determination of Precipitating Antibody Strength of Fortifying and Test Sera.*—A series of dilutions of a specific (fortifying serum) antiserum, which contains a high precipitin titer for the antigen, is made with saline. Each dilution is tested in a gel double diffusion tube against a given concentration of antigen. The gel double diffusion method used was described previously[5] with the following exceptions. Saline (0.9 per cent) adjusted to pH 8.0 with 1N NaOH was used in the preparation of the agar medium in place of the phosphate buffer. The column lengths of the antigen-agar and center reaction column should be measured carefully and were 4.5 and 0.5 cm., respectively. Place 0.5 ml. of each dilution into a prepared gel double diffusion tube and observe for visible precipitation after 48 hours of incubation. The greatest dilution which shows a visable precipitate band after 48 hours at 37° C. is determined. The test serum is treated in the same way, and the greatest dilution of this serum which gives a visible precipitation in 48 hours is the dilution to be used in the next step. If undiluted test serum fails to produce a visible precipitate in the gel double diffusion tube after 48 hours, the undiluted test serum is used in step 2.

2. *Preparation of Serum Mixtures.*—Prepare a second series of dilutions beginning the series with one half that dilution determined as an end point in step 1. Next, take 0.25 ml. of these dilutions and mix each with an equal volume of the test serum. After mixing the sera, the first tube will contain a dilution of fortifying serum equal to the greatest dilution which visibly precipitates in a gel double diffusion tube after 48 hours of incubation as determined previously, and each mixture will contain a uniform amount of test serum antibody in a total volume of 0.5 ml.

3. *The Gel Double Diffusion Reaction.*—Place 0.5 ml. of each mixture from step 2 into a prepared gel double diffusion tube and observe for visible precipitation after 48 hours of incubation. Record the last gel double diffusion tube of the series in which a visible precipitate is observed. Prepare a control series of tubes by substituting 0.9 per cent saline for the test serum in the serum mixtures.

4. *Interpretation of Serum Fortification Analysis.*—After the 48 hour incubation period, determine the gel double diffusion tube which is just positive for precipitation. Compare the known total dilution of fortifying serum of the mixture in the end point tube with that fortifying serum dilution present in the positive tube of the saline control set. If the difference in dilution of fortifying serum in the test end point tube and the saline control is zero, it can be assumed that no antibody is present in 0.25 ml. of the test serum. If the difference is greater than zero, the addition of 0.25 ml. of test serum has contributed some antibody to the mixture. The difference between the fortifying serum dilutions in the end point tubes of the test mixtures and the saline control set of gel double diffusion tubes is called the *differential titer.*

In effect, when a serial dilution titration of a serum is followed by serum fortification analysis, a double titration is achieved. In order to combine the serial dilution titration titer of the test serum with the differential titer from serum fortification and thereby obtain a single numerical value *(reactive antibody units)* which locates the test serum on a scale of relative reactive antibody strength, the following method is proposed. The differential titer is first divided by 10 and the quotient from this division is added to the product of the reciprocal of the initial dilution of the test serum used in the fortification procedure multiplied by 10. For example, if the test serum in a 1:2 dilution is determined by a serial dilution titration to give minimal gel double diffusion precipitation after 48 hours of incubation and use of this 1:2 dilution in a fortification analysis results in a differential titer of 370, the calculation of reactive antibody units is as follows:

$$370 \text{ (differential titer)} \div 10 = 37$$

$$37 + [2 \text{ (test serum dilution)} \times 10] = 57 \text{ reactive antibody units}$$

To illustrate the serum fortification technique in application an isolated experiment is presented in Table I. The test serum for this example was obtained from an individual with active tuberculosis and the undiluted serum failed to precipitate with antigen in the regular gel double diffusion test for mycobacterial antibody. A rabbit antiserum, which gave a minimal positive gel double diffusion test for mycobacterial antibody after 48 hours of incubation when a 1:20 dilution was used, was chosen as the fortifying serum. Eleven serial dilutions beginning with 1:10 were prepared with saline. As shown in Table I, 0.25 ml. of each dilution was used in preparing the test serum mixtures in tubes 1 through 11. A parallel set of mixtures which contained 0.25 ml. of fortifying serum dilutions plus an equal volume of saline served as a saline control set. After 48 hours' incubation the fourth tube in the test series showed the same minimal precipitation as the first tube of the saline control. All but the first tube of the saline control set were negative. Since the fourth tube contained a 1:60 dilution of fortifying serum and the dilution of the fortifying serum in tube 1 of the saline control was 1:20, a differential titer of 1:40 was obtained. This indicated that the test serum, in 50 per cent concentration, contributed sufficient homologous antibody to the 1:60 rabbit antiserum dilution to give it a reactive antibody strength equal to 1:20 dilution of the fortifying rabbit antiserum.

TABLE I. A TYPICAL FORTIFIED SERUM ANALYSIS OF A LOW TITERED TUBERCULOUS SERUM

GEL DOUBLE DIFFUSION TUBE	COMPOSITION OF SERUM MIXTURE			FORTIFYING* SERUM DILUTION IN THE MIXTURE	PRECIPITATION IN GEL DOUBLE DIFFUSION TUBES (48 HR.)
		*Fortifying serum**			
1	0.25 ml.	10	+ 0.25 ml. test serum	20	+
2		15		30	+
3		20		40	+
4		30		60	+
5		40		80	0
6		60		120	0
7		80		160	0
8		120		240	0
9		160		320	0
10		240		480	0
11		320		640	0
12†		10	+ 0.25 ml. NaCl	20	+

*Reciprocal of dilution.

†Actually tube No. 1 of saline control set. Tubes 2 to 11 of saline controls were negative after 48 hours of incubation.

If there had been no homologous antibody in the test serum, only the first tubes of the saline control and the test series would have been positive. By application of this calculation, this serum was evaluated for reactive antibody strength as follows:

$$40 \div 10 = 4$$
$$4 + (0 \times 10) = 4 \text{ reactive antibody units}$$

The results presented in this report were obtained from experiments in which two different supplies of immune rabbit antiserum were used. One serum, prepared in this laboratory according to a procedure given elsewhere[1, 2] against concentrated culture filtrate antigens from the mycobacterial strain H37Ra, was found to minimally precipitate in a gel double diffusion test after 48 hours' incubation when a 1:20 dilution was used. The second fortifying serum used was obtained commercially.* This serum in a 1:30 dilution gave minimal precipitation in a gel double diffusion tube after 48 hours' incubation. The 121 serum specimens used in this study were obtained from the Suburban Cook County Tuberculosis Hospital.† The nontuberculous sera were obtained from 150 healthy volunteers (54 of whom were skin test positive to 5 T.U. of purified protein derivative‡) and 8 subjects vaccinated with bacille Calmette Guérin (BCG) in the thirtieth postvaccination week.

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†Hinsdale, Ill.

‡Parke, Davis & Company, Detroit, Mich.

All antigens were unheated, concentrated culture filtrates obtained from 6-week-old pellicle cultures of the mycobacterial strain H37Ra grown on Youmans modified Proskauer and Beck medium.[6] The method used for preparation of the antigen has been given in an earlier report.[4]

RESULTS

For the first experiment, undiluted test sera which had failed to produce visible precipitates in gel double diffusion tubes after 48 hours of incubation, and test sera which had produced only slightly visible or no precipitates with undiluted sera and which were all negative when the serum was diluted 50 per cent with saline were analyzed by the fortified serum technique. Eighty-nine sera from tuberculous persons, sera from 150 healthy volunteers, and sera from 8 subjects vaccinated with BCG were included in this experiment. All specimens were analyzed using eleven dilutions of fortifying rabbit antiserum for preparation of the test mixtures. The dilutions were 1:10, 1:15, 1:20, 1:30, 1:40, 1:60, 1:80, 1:120, 1:160, 1:240, and 1:320. The results obtained from this analysis are given for the tuberculous sera in Table II. All nontuberculous serum specimens were found to be negative in both the fortified serum as well as the single tube gel double diffusion tests.

TABLE II. RESULTS OF FORTIFIED SERUM ANALYSIS OF 89 UNDILUTED LOW TITERED TUBERCULOUS SERUM SPECIMENS

FORTIFIED SERUM* IN TEST MIXTURES	NO. OF SPECIMENS WITH TEST END POINTS AT EACH DILUTION	DIFFERENTIAL TITERS FOR EACH DILUTION	CALCULATED REACTIVE UNITS
20	0	0	0
30	1	10	1
40	4	20	2
60	12	40	4
80	16	60	6
120	39	100	10
160	9	140	14
240	5	220	22
320	3	300	30
480	0	460	46
640	0	620	62
20 (positive saline control)	89	–	–

*Reciprocal of dilutions.

The results from this experiment demonstrate the merit of fortified serum analysis. If these 89 sera had been tested only by the regular gel double diffusion test for mycobacterial antibody, all would have been placed in either of two groups; seven would have been determined as negative for the antibody and the remainder as only faintly positive. With the fortified serum test, all 89 were found to contain some detectable antibody and the sera now fall into 8 separate groups with calculated reactive antibody units ranging from 1 to 30.

Upon finding that serum fortification accomplished such divisional spread, a second experiment was devised which consisted of a fortification analysis of 32 relatively high titered tuberculous serum specimens.

A commercially obtained rabbit antiserum in dilutions of 1:15, 1:20, 1:25, 1:50, 1:100, 1:125, 1:150, 1:200, 1:250, 1:400, and 1:500 was used to prepare the test mixtures. In the saline control tube, this fortifying serum precipitated antigen after 48 hours of incubation in a dilution of 1:30. The results from this experiment are presented in Table III. From these data, it is apparent that

TABLE III. RESULTS OF FORTIFIED SERUM ANALYSIS OF 32 RELATIVELY HIGH TITERED TUBERCULOUS SERUM SPECIMENS

NO. OF SPECIMENS	TITERS* BY GEL DOUBLE DIFFUSION TITRATION	NO. OF SERA	DIFFERENTIAL TITERS*	CALCULATED UNITS OF REACTIVE ANTIBODY
17	2	6	70	27
		9	170	37
		2	370	57
3	6	1	70	67
		2	170	77
3	16	2	70	167
		1	170	177
8	17	5	95	179.5
		2	170	187
		1	220	192
2	18	1	170	197
		1	370	217

*Reciprocals.

a simple dilution gel double diffusion titration gives a titer spread ranging from 1:2 to 1:18 in which 17 sera give minimal end point reactions in dilution of 1:2, 3 in a dilution of 1:6, 3 in a dilution of 1:16, 8 in a dilution of 1:17, and 2 in a dilution of 1:18. By comparison the addition of fortified serum analysis resulted in a much wider spread with calculated antibody strength values which ranged from 27 to 217. Furthermore, the represented number of groups to which each serum belongs was increased from 5 to 12. As will be pointed out in the discussion, this more accurate determination of antibody strength is of considerable value, especially in performance of immunologic procedures in chronic disease.

DISCUSSION

The serum fortification technique has been shown to be a very sensitive quantitative method for detecting slight differences in the antibody concentrations of different serum specimens. The tube gel double diffusion reaction is inherently sensitive.[7] Serum fortification enhances this sensitivity. The added increase is comparable to that which might be obtained if antibody in the test serum were concentrated by a means such as lyophilization. This effect of serum concentration in the gel double diffusion tube is achieved through the measured addition of a high titered homologous antibody serum to one of low precipitin strength. This addition results in a mixture of homologous antibody from two sources. The precipitin strength of one (the fortifying serum) is known and that of the other (the test serum) is unknown. If the mixture is prepared so that its precipitin strength is easily determined by gel double diffusion tube analysis, the difference between the precipitin strength of the mixture and the fortifying serum component represents the precipitin strength of the unknown test serum.

In general, any mechanism which tends to increase antibody concentration tends to counteract excess antigen effects both in vivo and in vitro. The consequent formation of a more readily visualized antigen-antibody precipitation renders the gel diffusion precipitation system more effective for the detection of antibody.

It should be mentioned that even though the serum fortification method provides precise data, the technique is very flexible and may be varied in several ways. If detection of even closer differences in antibody concentration than are demonstrated in this report are desired, instead of mixing the two sera in equal proportions, the ratios of test serum to fortifying serum in each mixture could be altered to accomplish this purpose. Literally, it is possible to tailor this method to suit the demands of an investigation by altering such factors as the nature of the fortifying dilution series, incubation time, and relative volumes of the various reagents.

The need for detecting very small differences in the amounts of antibody between two different serum specimens becomes apparent when the following concepts are considered. The in vitro demonstration of antibody does not depend solely upon the efficacy of antibody production by the body tissues. Precipitating antibody may be efficiently elaborated and still may be difficult to demonstrate by in vitro techniques.

The broad assumption may be made that the classic concepts of precipitating antigen-antibody systems apply to some extent in vivo. Furthermore, it may be assumed that if antigens are elaborated from organisms present in infected tissues, these antigens may enter the blood stream and various other body fluids. If this is true, and if antibody is also present, all relative concentrations of coexisting serum antigen and antibody are possible. Such coexisting serum antigen and antibody concentrations may give rise to any one of the following possible in vivo situations: (1) all antigen and antibody may precipitate completely; (2) if there is a sufficient excess of antigen, it may combine with antibody as the soluble complex; (3) if antibody is in excess, all antigen may be completely precipitated, but at the expense of circulating free antibody.

The first situation described would result in a negative in vitro test for precipitating antibody as no free serum antibody would be available. In the second situation, an in vitro test would be negative also, unless the situation of excess antigen were relieved by increasing the antibody per volume content of the serum, since antigen excess tends to inhibit the formation of visible precipitates. With all in vitro immunologic procedures in which antigen is added to the system the condition of the antigen excess in the serum is aggravated. This might similarly be the case with the serum from situation 3, unless the antibody appeared in considerable excess of the combined concentration of in vivo and in vitro antigen. Thus, even in some sera which would be classified as representative of the third situation, antigen excess inhibition in vitro remains a possibility.

The fortification technique tends to counteract in vitro excess antigen effects, since it is the antibody content of the immunologic system which is strengthened.

In considering the concepts and situations presented it would seem that in vitro demonstration of even very minute differences in antibody titers might be of considerable importance in the immunologic study of chronic disease serum.

SUMMARY

Serum fortification analysis, a new immunologic technique useful for the quantitative measurement of small difference in antibody titers of different serum specimens has been presented. This technique employs the use of a serum of known high concentration of homologous antibody which when mixed in graded amounts with a constant amount of low titered serum serves to fortify the serum of lesser antibody strength. By measuring the difference between the precipitating strength of the serum mixtures and the strength of the fortifying serum, the antibody concentration of the test serum can be accurately determined. The technique used for the demonstration of precipitins in this report was a modified Oakley gel double diffusion precipitation method.

Data obtained through the analysis of serum specimens from tuberculous, nontuberculous, and BCG vaccinated individuals have been presented. All of the tuberculous sera were found to possess a measurable amount of antibody by this method, whereas none of the other serum specimens contained detectable specific precipitins. That this new immunologic technique might be of value in undertaking studies of antibody response in patients with chronic diseases has been indicated.

REFERENCES

1. Parlett, R. C., and Youmans, G. P.: Antigenic Relationships Between Mycobacteria as Determined by Agar Diffusion Precipitation Techniques, Am. Rev. Tuberc. **73**: 637, 1956.
2. Parlett, R. C., and Youmans, G. P.: Antigenic Relationships Between Ninety-eight Strains of Mycobacteria Using Gel Diffusion Precipitation Techniques, Am. Rev. Tuberc. **77**: 450, 1958.
3. Parlett, R. C., Youmans, G. P., Rehr, C., and Lester, W.: The Detection of Antibodies in the Serum of Tuberculous Patients by an Agar Double-Diffusion Precipitation Technique, Am. Rev. Tuberc. **77**: 462, 1958.
4. Parlett, R. C., and Youmans, G. P.: An Evaluation of the Specificity and Sensitivity of a Gel Double Diffusion Test for Tuberculosis, Am. Rev. Resp. Dis. **80**: 153, 1959.
5. Parlett, R. C., and Rehr, C. A.: Further Studies on Gel Diffusion Tests in Tuberculosis. I. Methods for Standardization of the Antigen and Detection of Small Amounts of Antibody, Am. Rev. Resp. Dis. **80**: 886, 1959.
6. Youmans, G. P., and Karlson, A. G.: Streptomycin Sensitivity of Tubercle Bacilli: Studies on Recently Isolated Tubercle Bacilli and Development of Resistance to Streptomycin in Vivo, Am. Rev. Tuberc. **55**: 529, 1947.
7. Wilson, M. W.: The Agar Diffusion Precipitation Technique; A Comparison of Simple and Double Diffusion Methods, J. Immunol. **81**: 317, 1958.

SOME USEFUL PROCEDURES FOR GROUP DIFFERENTIATION OF MYCOBACTERIUM TUBERCULOSIS, UNCLASSIFIED MYCOBACTERIA, AND SAPROPHYTIC ACID-FAST BACILLI

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Interest in pulmonary disease and other infections caused by unclassified mycobacteria has been greatly intensified in recent years. Because such organisms are often difficult to distinguish from tubercle bacilli and other acid-fast bacteria, investigators have been developing procedures to aid in the identification of these organisms.

The investigation described was undertaken to evaluate further the usefulness of several of these tests for group differentiation of various mycobacteria, to study certain improvements in their methodology, and to summarize the interpretation of the results.

All these tests which are described in detail are efficient, easy to perform, may readily be adapted in almost any laboratory, and have been found useful for the purpose described.

IN PREVIOUS studies[1-5] it was observed that the thioglycollate, peroxidase, neutral red, serpentine cord, and niacin tests were useful for group differentiation of human tubercle bacilli, unclassified mycobacteria, and saprophytic acid-fast bacilli. Because of the communications which we have received inquiring about various details of these procedures and because of recent improvements in methodology, it was considered worth while in this study not only to present the technique of each test in detail as used in our laboratory but to summarize the interpretation of the results. Included in this report are a number of bovine, avian, and Battey strains of mycobacteria which have not been studied previously.

MATERIALS AND METHODS

Fifty strains of freshly isolated human, 2 strains of bovine, and 2 strains of avian tubercle bacilli, 50 strains of unclassified mycobacteria, and 5 strains of saprophytic acid-fast bacilli were selected for study. The sources of these organisms have been indicated previously.[3-5] They were transferred to Löwenstein-Jensen and Proskauer-Beck media[6] to maintain stock cultures.

Thioglycollate Test.[1-4]—

Procedure[3]*:* 1. Tubes of fluid thioglycollate medium† were inoculated with one loopful of each test organism which had grown for approximately 7 days in Tween-albumin liquid

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†Thioglycollate medium without indicator, Brewer modified. No. 01-135-C, Baltimore Biological Laboratory, Baltimore, Md. The medium was prepared exactly as directed.

medium[5] or with the tip of a platinum wire which had been dipped into the bacterial masses of similar aged organisms growing on Löwenstein-Jensen medium.

2. The inoculated media were incubated for 8 weeks at 37° C.

3. The results were read daily during the entire period of incubation. Thioglycollate-positive strains grow in the medium, whereas negative strains do not.

Controls: Tubes of uninoculated media and known thioglycollate-positive and -negative strains were used as controls.

Peroxidase Test.[7, 8]—

Procedure: 1. One milliliter of a 2 per cent weight per volume aqueous solution of catechol and 0.5 ml. of a 1 per cent volume per volume aqueous solution of hydrogen peroxide were placed into each of a series of small screw-cap tubes and inoculated with two large colonies from each of the cultures.* .

2. The tubes were then incubated for 24 hours at room temperature (approximately 20° C.).

3. The results were read after 10 minutes, and again after 1, 12, and 24 hours, respectively. Peroxidase-positive strains turn brown within 10 minutes, darken, and become black within 1 to 24 hours. Negative strains remain unchanged.

Controls: Known peroxidase-positive and -negative strains were used as controls.

Neutral Red Cytochemical Test.[9-15]—

Procedure[12]*:* 1. Five milliliters of 50 per cent volume per volume methanol was placed into each of a series of small screw-cap tubes and was inoculated with two large colonies from each of the cultures.

2. The tubes were incubated for 1 hour at 37° C., then centrifuged to pack the organisms, and the alcohol was decanted.

3. Five milliliters of the alcohol was again added to each tube, the organisms were resuspended by shaking the tubes, and step 2 was repeated.

4. Five milliliters of alkaline-barbiturate buffer† and 0.2 ml. of a 0.05 per cent weight per volume aqueous solution of neutral red were added to each tube, the contents were shaken, and the tubes were incubated for 1 hour at 37° C.

5. The tubes were shaken thoroughly every 15 minutes and the results were read after the period of incubation. Neutral red positive strains exhibit varying degrees of pink or red staining of the organisms. Negative strains remain unchanged.

Controls: Known virulent, attenuated, and avirulent strains were used as controls.

Serpentine Cord Test.[10, 13-22]—

Procedure[18]*:* 1. The test strains were grown in Tween-albumin liquid medium[5] containing 0.05 per cent Tween 80, 0.5 per cent bovine albumin, and 5 per cent human plasma which had not been subjected to heat.

2. The cultures were incubated for 14 days at 37° C.

3. By means of serologic or Pasteur pipettes and with as little agitation as possible, smears from the cultures were made by adding one drop of water to each slide before transferring the culture material to it.

4. The smears were dried, fixed, and acid-fast stained by the Ziehl-Neelsen technique.

5. The smears were examined under the high dry and oil immersion objectives. Positive strains exhibit varying degrees of "serpentine" cording, whereas negative strains are noncorded.

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*When there was no further need to save the cultures, some of the tests also were performed directly on the cultures by flooding the bacterial mass in each tube with the reagents.

†Buffer: Sodium barbital, 1.0 Gm., sodium chloride, 5.0 Gm., and distilled water, 100 ml.

Controls: Known virulent, attenuated, and avirulent strains were used as controls.

Micro-Niacin Test.[5, 23-29]—

Procedure[5]*:* Water extracts were prepared in the following manner:

1. From 2 to 10 drops of distilled water was added to each culture* depending on the amount of fluid already present.

2. The tubes were placed in a nearly horizontal position for approximately 5 minutes to enable the fluid to cover the entire surface of the bacterial mass and thus permit greater extraction of niacin.

3. After this time, the extracts from each culture were removed by means of separate 8 inch disposable plastic straws fitted with a removable rubber aspirating bulb and placed into 10 by 100 mm. serologic tubes.

4. The extracts were sterilized for 15 minutes at 15 pounds' pressure and cooled to room temperature. Niacin tests were then performed by the following method.

Micro-plate test.—

Procedure: 1. Two drops of extract from each strain was removed from the serologic tubes by means of separate plastic straws and placed into appropriately marked depressions in white glazed porcelain spot plates.

2. To each extract were added 1 drop of 4 per cent volume per volume aniline in 95 per cent ethanol and 1 drop of a 10 per cent weight per volume aqueous solution of cyanogen bromide. The contents were mixed by sliding the plates back and forth in a rotary motion, care being taken to prevent spillage.

3. The results were read approximately 5 minutes after the reagents were mixed with the extracts by means of a reflected white or daylight source. Water extracts of niacin-positive strains exhibit varying degrees of yellow coloration. Extracts of negative strains remain unchanged.

Controls:

Reagent Control.—One drop of each reagent was added to a depression in a spot plate, mixed, and allowed to stand for approximately 5 minutes. The mixture should be colorless.

Medium Control.—From 2 to 10 drops of distilled water was added to a tube of the uninoculated medium depending on the amount of fluid already present and a niacin test was performed on the water blank in exactly the same manner as for the inoculated medium.

Pigmented Strain Controls.—Pigmented strains which colored the water extracts also were compared with the following two controls: (a) 2 drops of extract, 1 drop of aniline reagent, and 1 drop of distilled water, and (b) 2 drops of extract, 1 drop of cyanogen bromide reagent, and 1 drop of water.

Test Strain Controls.—Known niacin positive, doubtful, and negative strains also were included.

All hazardous procedures were carried out under a well-ventilated bacteriologic safety hood. Each test was repeated two times. The final readings represent average results.

RESULTS

The data presented in Table I summarize typical findings from each group of organisms. The thioglycollate medium failed to support the growth of any of the tubercle bacilli, except the Sheard avian strain which grew poorly. The

*All the organisms were grown on the Tween-albumin niacin test medium[5] which was inoculated with 0.3 ml. aliquots of the bacterial suspensions prepared by the culture and dilution method described previously.[5] Cultures 14 days old were used to perform the niacin tests.

TABLE I. RESULTS OF THIOGLYCOLLATE, PEROXIDASE, NEUTRAL RED, SERPENTINE CORD, AND NIACIN TESTS WITH M. TUBERCULOSIS, UNCLASSIFIED MYCOBACTERIA, AND SAPROPHYTIC ACID-FAST BACILLI

STRAIN	T TEST	P TEST	NR TEST	SC TEST	N TEST
Human tubercle bacilli					
A-1	–	+	+	+	+
A-2	–	+	+	+	+
A-3	–	+	+	+	+
A-4	–	+	+	+	+
A-5	–	+	+	+	+
A-6	–	+	+	+	+
A-7	–	+	+	+	+
A-8	–	+	+	+	+
A-9	–	+	+	+	+
A-10	–	+	+	+	+
H37Rv	–	+	+	+	+
H37Ra	–	+	–	–	+
Bovine tubercle bacilli					
Copenhagen	–	+	+	+	–
Ravenel	–	+	+	+	+
Avian tubercle bacilli					
Kirchberg	–	–	–	–	–
Sheard	2+	–	–	–	–
Unclassified mycobacteria photochromogens					
AP-1	28+	–	–	–	–
AP-2	27+	–	–	–	–
AP-7	11+	–	–	–	–
AP-9	6+	–	–	–	–
AP-10	–	–	–	–	–
AP-11	–	–	–	+	–
Scotochromogens					
AS-1	39+	–	–	–	–
AS-2	28+	–	–	–	–
AS-12	13+	–	–	–	–
AS-14	6+	–	–	–	–
AS-15	4+	–	–	–	–
AS-16	–	–	–	–	–
Battey					
RB-1	4+	+	+	–	–
RB-2	3+	+	+	–	–
RB-3	3+	+	+	–	–
RB-4	–	–	–	–	–
AB-7	3+	–	–	–	–
AB-8	2+	–	–	+	–
Saprophytic mycobacteria					
M. smegmatis-a	1+P	–	–	–	–
M. smegmatis-b	1+P	–	–	–	–
M. phlei-a	1+P	–	–	+	–
M. phlei-b	1+P	–	–	+	–
M. butyricum	1+P	–	–	–	–

P = pellicle formation. Numbers = the day growth was first observed.

medium also supported the growth of 78 per cent of the unclassified mycobacteria (4 photochromogens, 5 scotochromogens, and 5 Battey types) and 100 per cent of the saprophytes. In most instances, growth of the unclassified mycobacteria was slow and dysgonic, requiring from 6 to 28 days for initial development of the photochromogens, from 4 to 39 days for the scotochromogens, and from 2 to 4 days for the Battey organisms. The saprophytes grew rapidly and luxuriantly within 1 day with characteristic pellicle formation (Fig. 1).

All the human tubercle bacilli gave positive results with the peroxidase, neutral red, serpentine cord, and niacin tests except the avirulent H37Ra strain which gave negative neutral red and serpentine cord tests. The bovine tubercle bacilli gave positive results with the peroxidase, neutral red, and cord tests. The niacin tests were negative with the Copenhagen strain, and only slightly positive with the Ravenel strain. The avian tubercle bacilli were negative with each of these four tests.

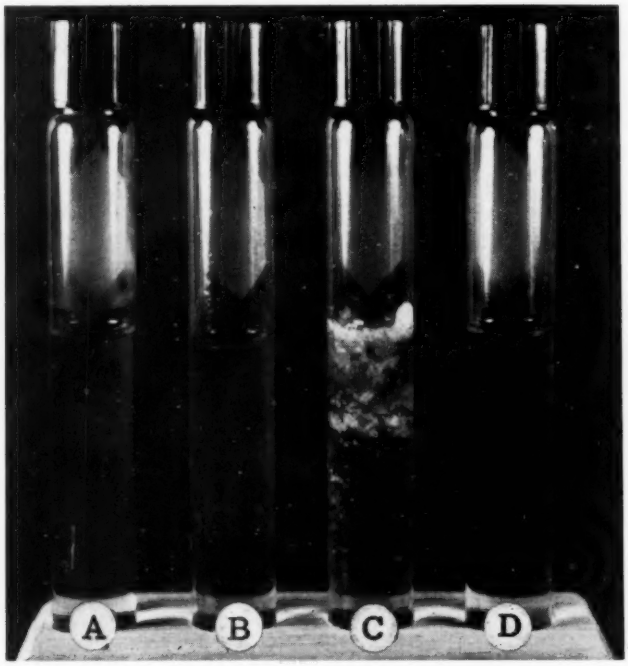

Fig. 1.—Photograph of some typical thioglycollate test results. *A*, Negative test, strain H37Rv, a virulent human tubercle bacillus. No growth. *B*, Positive test, strain AS-12, a scotochromogenic unclassified Mycobacterium. Growth is slow and dysgonic requiring approximately 13 days for initial development. Pellicle formation is not observed. *C*, Positive test, strain *Mycobacterium smegmatis*, a saprophytic acid-fast bacillus. Growth is rapid and luxuriant within 1 day with characteristic pellicle formation. *D*, Control, uninoculated medium.

With eight exceptions, all of the unclassified mycobacteria also gave negative results with each of these four tests. Three of the Battey organisms gave both a positive peroxidase and neutral red test, but the reactions were slight compared to those obtained with the tubercle bacilli. One of the photochromogens and one of the Battey strains exhibited partial cording (Fig. 2).

All of the saprophytic organisms also gave negative results with each of these four tests except the *Mycobacterium phlei* strains which showed partial cording.

DISCUSSION

Although, in most instances, the peroxidase, neutral red, serpentine cord, and niacin tests are able to distinguish the human and bovine tubercle bacilli from the avian, unclassified, and saprophytic mycobacteria, only the peroxidase

and neutral red tests are able to differentiate some of the Battey strains from the other unclassified organisms, the avian, and saprophytic mycobacteria. With minor exceptions, the serpentine cord and niacin tests can not differentiate

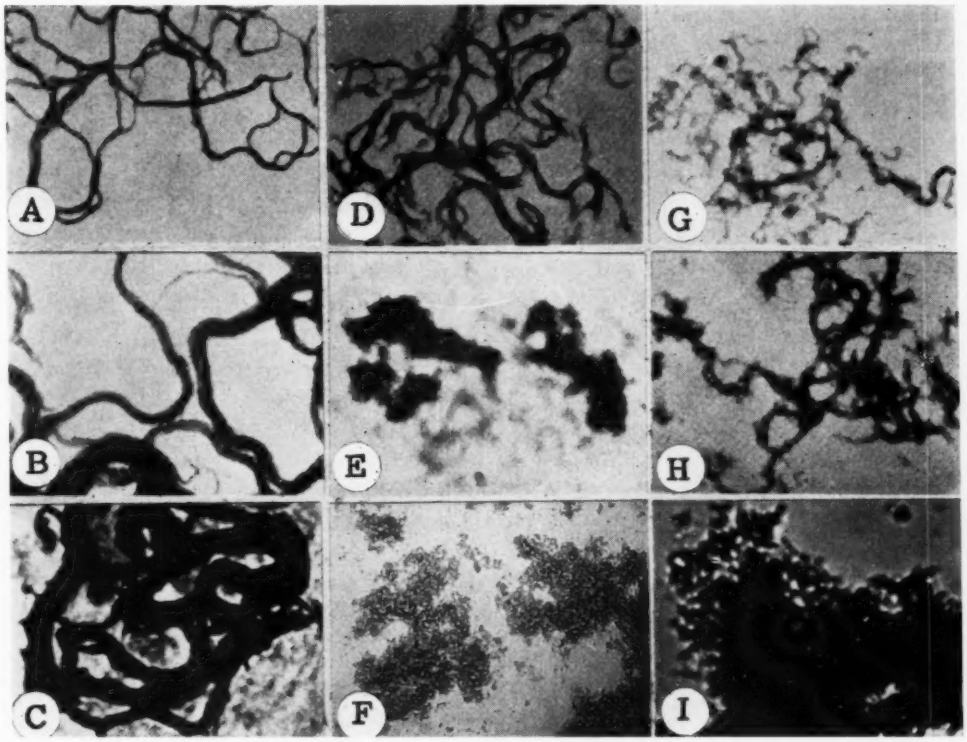

Fig. 2.—Photomicrographs of some typical serpentine cord test results. Ziehl-Neelsen stained smears of 7-day-old cultures in Tween-albumin liquid medium containing 0.05 per cent Tween 80, 0.5 per cent bovine albumin, and 5 per cent unheated human plasma.

A, Intensely positive test, strain H37Rv, a virulent human tubercle bacillus showing characteristic serpentine cording. (Magnification × 430.) *B* and *C*, Intensely positive tests, larger magnification of strain H37Rv. Note the compact parallel cellular orientation. (Magnification × 970.) Strains of intermediate virulence exhibit varying degrees of cording. *D*, Intensely positive test, strain Ravenel, a virulent bovine tubercle bacillus also exhibiting characteristic cording. (Magnification × 430.) *E*, Negative test, strain BCG (Phipps), an avirulent bovine tubercle bacillus. Note the amorphous clumping and lack of cellular orientation. (Magnification × 430.) This type of structure is characteristic of most avirulent mycobacteria. *F*, Negative test, strain Sheard, a virulent avian tubercle bacillus showing amorphous clumping and lack of cellular orientation. (Magnification × 430.) Although virulent, the avian organism does not form serpentine cords. *G*, Slightly positive test, strain P-1, an unclassified photochromogenic mycobacterial pulmonary pathogen exhibiting poor parallel cellular orientation. (Magnification × 430.) For comparative purposes, the organism was graded as slightly positive. Typical serpentine cording is not characteristic of these strains. *H*, Slightly positive test, strain *M. phlei*, a saprophytic Mycobacterium showing some cording, but lacking marked parallel cellular orientation. (Magnification × 430.) Other saprophytic mycobacteria may exhibit varying degrees of cording under similar conditions, but true cording does not occur. *I*, Negative test, strain *M. smegmatis*, another saprophytic acid-fast bacillus showing amorphous clumping and lack of serpentine cellular orientation. (Magnification × 970.)

the avian, unclassified, and saprophytic organisms. On the other hand, in most instances, the thioglycollate test is able to distinguish the human and bovine tubercle bacilli from the other groups of mycobacteria.

Despite the fact that the peroxidase and neutral red tests were not as useful as the other procedures for differentiating human and bovine tubercle

bacilli from the Battey strains, they proved equally as helpful as the other tests in distinguishing these tubercle bacilli from the other groups of organisms.

Although a number of the Battey strains (50 per cent) were neutral red–positive, the reactions, in general, were never as intense as those obtained with virulent human and bovine tubercle bacilli, nor did the Battey organisms retain the neutral red for as long as the tubercle bacilli. For example, if the tests were left to stand for 24 hours, it was observed that, whereas all of the tubercle bacilli appeared to retain about the same intensity of coloration observed immediately after reading the results (1 hour), the majority of the Battey strains had either faded in varying degrees or lost their red color completely.

The peroxidase reactions exhibited by the Battey organisms also were less marked than those obtained with the tubercle bacilli. Peroxidase is a cytochrome enzyme present in large amounts in isoniazid-susceptible human and bovine tubercle bacilli, but deficient in avian tubercle bacilli, unclassified mycobacteria, and saprophytic acid-fast bacilli. Catechol is oxidized to *o*-benzoquinone which condenses to form brown compounds of uncertain composition. The production of the brown color has been taken as an index of enzymatic activity. Mycobacteria made resistant to isoniazid lose their ability to split hydrogen peroxide to molecular oxygen. The enzyme is not diminished in strains resistant to streptomycin, para-aminosalicylic acid, or amithiozone.[7] Therefore, isoniazid-resistant mycobacteria will give negative or weakly positive peroxidase reactions depending on the degree of drug resistance. This factor must be kept in mind when using the peroxidase test. It also emphasizes the limitations of any one differential test and the importance of employing more than one procedure in diagnostic work.

Although several of the unclassified and saprophytic mycobacteria exhibited varying degrees of cording, this was observed only in portions of the smears. The majority of the bacilli were disoriented and formed amorphous clumps which is characteristic of avirulent acid-fast bacilli (Fig. 2). The ability of certain mycobacteria to form varying degrees of cording has been reported previously.[3, 10, 14, 21] Keeping in mind that true cording, such as exhibited by virulent tubercle bacilli, is the ability of an organism to form serpentine cords of varying thickness and length consisting of strongly acid-fast bacilli oriented in parallel along the cords, careful observation should, in most instances, enable one to differentiate true from pseudocording. Mention also should be made of the reports of Hoyt and co-workers[19] and Goldman and Goldman[20, 21] who demonstrated that mycobacteria may exhibit varying degrees of cording depending on the medium, size of the inoculum, incubation time, and the presence of bacterial contamination. Accordingly, these factors must be kept in mind when performing the serpentine cord test.

When used in conjunction with the serpentine cord test, the neutral red test proved helpful in determining the virulence of many of these mycobacteria. The high level of agreement between the neutral red and animal virulence tests has been demonstrated.[10–12] By the use of membrane filters, Wayne[13, 15] and

Krasnow and associates[14] were able to differentiate various strains of mycobacteria by a combined neutral red and serpentine cord test. The method is more elaborate than the tests described here and greater care in detail must be adhered to for its successful use.

On prolonged incubation, the thioglycollate medium may form flocculent or crystalline precipitates. This is due to concentration and may simulate growth. The crystalline material is probably mainly cystine, and the flocculent matter largely agar. With some experience one can soon learn to distinguish this from growth. When shaking the tubes, these precipitates can usually be dispersed and are no longer prominently visible. The medium may show varying degrees of uniform opalescence depending on the amount of precipitate present. Growth may also be dispersed, but the medium appears more opaque and bacterial matter is usually observed. If there is doubt, smears and subcultures can be made and compared with control smears and subcultures prepared immediately after the medium is inoculated and before it is incubated. Thus far, the type of thioglycollate indicated has given the best results. It also is recommended that only fresh batches of medium be used.

Despite various reports describing the limitations of the direct qualitative Konno test for niacin, recent improvements in methodology have helped overcome these difficulties. The improved techniques are now not only more efficient, but safer and more economical than some of the procedures described previously.

Although many other useful differential tests have been reported, those described here were selected for study primarily because of their simplicity, efficiency, and ready adaptability in almost any laboratory. As more of these types of organisms are isolated and studied, and with the continuing advances in this field of investigation, other and no doubt better techniques will be developed.

SUMMARY

The investigation described here was undertaken to evaluate further the usefulness of several tests for group differentiation of various mycobacteria, to study certain improvements in their methodology, and to summarize the interpretation of the results.

From these findings it can be seen that, in most instances, the peroxidase, neutral red, serpentine cord, and niacin tests are able to distinguish the human and bovine tubercle bacilli from avian, unclassified, and saprophytic mycobacteria, but only the peroxidase and neutral red tests are able to differentiate some of the Battey strains from the other unclassified organisms, the avian, and saprophytic mycobacteria. With minor exceptions, the serpentine cord and niacin tests cannot differentiate between the avian, unclassified, and saprophytic organisms. On the other hand, in most instances, the thioglycollate test is able to distinguish the human and bovine tubercle bacilli from the other groups of mycobacteria, and when used in conjunction with the other tests, differentiation of these organisms is further facilitated.

Although the peroxidase and neutral red tests were not as useful as the other procedures for differentiating human and bovine tubercle bacilli from the Battey strains, they proved equally as helpful as the other tests in distinguishing these tubercle bacilli from the other groups of organisms. Despite the fact that a number of Battey organisms gave positive peroxidase and neutral red tests, in most instances the reactions were less marked than those obtained with the human and bovine strains. When used in conjunction with the serpentine cord test, the neutral red test proved helpful in determining the virulence of many of these mycobacteria.

All these tests are easy to perform, and have proved useful for group differentiation of *Mycobacterium tuberculosis,* unclassified mycobacteria, and saprophytic acid-fast bacilli.

REFERENCES

1. Tarshis, M. S., and Frisch, A. W.: Chromogenic Acid-Fast Bacilli From Human Sources. I. Cultural Studies, Am. Rev. Tuberc. **65:** 278, 1952.
2. Koch, M. L., Griffin, V. L., and Agostini, E. E.: The Selective Activity of Fluid Thioglycollate Medium for Group Differentiation of Atypical Chromogenic Mycobacteria, Mycobacterium tuberculosis, and Saprophytic Mycobacteria, Am. Rev. Tuberc. **77:** 356, 1957.
3. Tarshis, M. S.: Further Investigation on the Selective Activity of Fluid Thioglycollate Medium for Group Differentiation of M. tuberculosis, Anonymous (Atypical) Acid-Fast Bacilli, and Saprophytic Mycobacteria, J. Lab. & Clin. Med. **54:** 630, 1959.
4. Tarshis, M. S.: Thioglycollate, Peroxidase, Neutral Red, Serpentine Cord, and Niacin Tests for Group Differentiation of M. tuberculosis, Anonymous (Atypical) Acid-Fast Bacilli, and Saprophytic Mycobacteria, Dis. Chest. **38:** 413, 1960.
5. Tarshis, M. S.: A Direct Qualitative Micro-niacin Test for Differentiating Human Tubercle Bacilli From Other Mycobacteria. A Modified Procedure, Transactions of the Nineteenth Conference on the Chemotherapy of Tuberculosis, Veterans Administration–Armed Forces, 1960, p. 236; Am. Rev. Respir. Dis. **82:** 82, 1960.
6. Tuberculosis Laboratory Methods, Veterans Administration–Armed Forces Cooperative Study on the Chemotherapy of Tuberculosis, 1958, pp. 28-29.
7. Tirunarayanan, M. O., and Vischer, W. A.: Relationship of Isoniazid to the Metabolism of Mycobacteria, Am. Rev. Tuberc. **75:** 62, 1957.
8. Bogen, E.: Problems in Laboratory Diagnosis of Tuberculosis, Am. Rev. Tuberc. **76:** 1110, 1957.
9. Dubos, R. J., and Middlebrook, G.: Cytochemical Reaction of Virulent Tubercle Bacilli, Am. Rev. Tuberc. **58:** 698, 1948.
10. Richmond, L., and Cummings, M. M.: An Evaluation of Methods of Testing the Virulence of Acid-Fast Bacilli, Am. Rev. Tuberc. **62:** 632, 1950.
11. Morse, W. C., Dail, M. C., and Olitzky, I.: A Study of the Neutral Red Reaction for Determining the Virulence of Mycobacteria, Am. J. Pub. Health **43:** 36, 1953.
12. Hughes, D. E., Moss, E. S., Hood, M., and Henson, M.: Virulence of Mycobacterium Tuberculosis. Evaluation of a Test, Using Neutral Red Indicator, Am. J. Clin. Path. **24:** 621, 1954.
13. Wayne, L. G.: Cultivation and Visualization of Mycobacteria on Molecular Filter Membranes, J. Bact. **69:** 92, 1955.
14. Krasnow, I., Wayne, L. G., and Salkin, D.: A Microcolonial Test for the Recognition of Virulent Mycobacteria, Am. Rev. Tuberc. **71:** 361, 1955.
15. Wayne, L. G.: Modified Microcolonial Test for Virulent Mycobacteria, Am. Rev. Tuberc. **73:** 600, 1956.
16. Middlebrook, G., Dubos, R. J., and Pierce, C. H.: Virulence and Morphological Characteristics of Mammalian Tubercle Bacilli, J. Exper. Med. **86:** 175, 1947.
17. Bloch, H.: Studies on Virulence of Tubercle Bacilli; Isolation and Biological Properties of Constituent of Virulent Organisms, J. Exper. Med. **91:** 197, 1950.
18. Yegian, D., and Kurung, J.: Growth Pattern and Virulence of Tubercle Bacilli, Am. Rev. Tuberc. **65:** 181, 1952.
19. Hoyt, A., Smith, C. R., and Gribkoff, G. P.: A Filter Paper Culture Technique for Early Detection of Microcolonies of Tubercle Bacilli, Am. Rev. Tuberc. **70:** 916, 1954.

20. Goldman, E. C., and Goldman, D. S.: The Induction of Cording in an Avirulent Variant of Mycobacterium Tuberculosis, J. Bact. **70**: 353, 1955.
21. Goldman, E. C., and Goldman, D. S.: Cording and Neutral Red Reaction of Two Avirulent Strains of Mycobacterium, Am. Rev. Tuberc. **73**: 674, 1956.
22. Melvin, I. G., and Vandiviere, H. M.: The Titration of Cord Formation as Related to Virulence of Mycobacteria, Am. Rev. Tuberc. **78**: 83, 1958.
23. Konno, K.: New Chemical Method to Differentiate Human-Type Tubercle Bacilli From Other Mycobacteria, Science **124**: 985, 1956.
24. Konno, K., Kurzmann, R., and Bird, K. T.: The Metabolism of Nicotinic Acid in Mycobacteria. A Method for Differentiating Tubercle Bacilli of Human Origin From Other Mycobacteria, Am. Rev. Tuberc. **75**: 529, 1957.
25. Konno, K., Kurzmann, R., Bird, K. T., and Sbarra, A.: Differentiation of Human Tubercle Bacilli From Atypical Acid-Fast Bacilli. I. Niacin Production of Human Tubercle Bacilli and Atypical Acid-Fast Bacilli, Am. Rev. Tuberc. **77**: 669, 1958.
26. Konno, K., Kurzmann, R., Bird, K. T., and Sbarra, A.: Differentiation of Human Tubercle Bacilli From Atypical Acid-Fast Bacilli. II. Clinical Application, Am. Rev. Tuberc. **77**: 675, 1958.
27. Konno, K., and Sbarra, A. J.: Differentiation of Human Tubercle Bacilli From Atypical Acid-Fast Bacilli. III. Modification of the Niacin Test, Using Tween-Albumin Liquid Medium, Am. Rev. Tuberc. **79**: 810, 1959.
28. Gilani, S., and Selkon, J. B.: The Niacin Test for Differentiating Human Tubercle Bacilli From Other Mycobacteria, Tubercle **39**: 396, 1958.
29. Runyon, E. A., Selin, M. J., and Harris, H. W.: Distinguishing Mycobacteria by the Niacin Test. A Modified Procedure, Am. Rev. Tuberc. **79**: 663, 1959.

DETECTION OF URINARY CATALASE BY DISK FLOTATION

ABRAHAM I. BRAUDE, M.D., AND HERBERT BERKOWITZ
PITTSBURGH, PA.

Observations with the disk flotation method indicate that catalase is absent from the urine of normal persons and present in urine from patients with diseases of the urinary tract, including certain forms of renal injury accompanied by otherwise normal urine.

PRELIMINARY studies have indicated that catalase appears in the urine only in diseases of the urinary tract and suggested that the catalase test would be a simple rapid method for bedside detection of abnormal urine.[1] Catalase would be expected to appear in infected urine because the enzyme occurs in most bacteria that attack the urinary tract (Table I) and in inflammatory exudates.[6] Because it is also present in very high concentration in the kidney and its cell-free extracts,[2] there is reason to expect catalase activity in the urine of patients with other forms of kidney injury.

Catalase was at first determined by mixing urine with equal parts of 3 per cent H_2O_2 and observing the bubbles of oxygen.[1] This technique was replaced by the paper disk flotation method of Gagnon and collaborators[3] when it was found that bubbles sometimes developed from other causes than decomposition of H_2O_2. Examination of the urine from many sick and healthy persons by disk flotation has substantiated the earlier impression and demonstrates its value as a simple procedure for distinguishing between normal and abnormal urine.

MATERIALS AND METHODS

Urine specimens from 130 hospitalized women were obtained with strict aseptic precautions by catheterization. The entire area surrounding the urinary meatus was thoroughly cleansed to the labia majora first with sterile cotton balls soaked in Phisohex (a preparation containing 3 per cent hexachlorophene) and then with cotton balls soaked in 1:2,000 benzalkonium chloride. Cultures of the cleansed area were made with cotton swabs inoculated onto blood agar plates before and after treatment with Phisohex and benzalkonium chloride and indicated that this procedure was extremely effective in removing bacteria and thereby eliminating the danger of introducing infection into the bladder. Midstream urine was collected from 100 male patients after treating the glans penis similarly with Phisohex and benzalkonium chloride. Immediately after collection the urine was cultured by the pour plate method of Sanford[4] for determining the number of viable bacteria. The sediment from 10 ml. of centrifuged urine was inoculated onto eosin methylthionine chloride agar and blood agar and into thioglycollate and trypticase soy broth for isolation and identification of the bacterial species. The urinary sediment was also examined for cells, casts, and bacteria in wet coverslip preparations and in smears stained by the Gram and Wright methods.

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The catalase test was performed by the method of Gagnon and co-workers.[3] Paper disks, No. 507-GH,* were specifically prepared from purest, highly absorbent paper for the assay of enzymes, catalysts, and other substances. The disks were held by sharp pointed tweezers, dipped into the urine and then dropped into test tubes measuring 16 mm. in diameter. After adding 5 ml. of 3 per cent hydrogen peroxide solution to the tube, the catalase activity was measured by recording the time required up to one hour for the disk to rise to the surface of the solution of hydrogen peroxide at room temperature. Flotation of the disk depends on the liberation of oxygen through the action of catalase on hydrogen peroxide ($2H_2O_2 \longrightarrow 2H_2O + O_2$), and the speed of flotation corresponds directly to the amount of catalase activity.

TABLE I. CATALASE ACTIVITY OF 18 HOUR BROTH CULTURES OF DIFFERENT BACTERIAL SPECIES

NO. OF STRAINS	STRAINS	DISK FLOTATION TIME (SEC.)	
		AVERAGE	RANGE
2	*Pseudomonas aeruginosa*	2.9	(2.7–3.2)
1	*Proteus morganii*	3.3	
2	*Proteus vulgaris*	4.15	(4.1–4.2)
2	*Proteus mirabilis*	4.2	(3.9–4.5)
3	*Staphylococcus aureus*	14.0	(13.4–14.7)
4	*Escherichia coli*	55.7	(5.13–60.0)
3	*Klebsiella pneumoniae*	58.1	(55.4–60.0)
1	*Aerobacter aerogenes*	88.0	

RESULTS

Normal Controls.—Normal urine specimens were obtained from seventy healthy men, age 17 to 48 years, with no preliminary cleansing of the glans penis. None of these 70 normal urine specimens possessed catalase activity. Large numbers ($> 100{,}000$) of coagulase-negative staphylococci were cultured from 4 specimens that gave negative catalase tests. These were shown to be contaminants because sterile urine cultures were obtained from these subjects after cleansing the glans penis with Phisohex and benzalkonium chloride.

TABLE II. INCIDENCE OF CATALASE REACTIONS (DISK METHOD) IN INFECTED URINE

BACTERIAL SPECIES	NO. OF INFECTED URINE SPECIMENS	NO. OF POSITIVE CATALASE REACTIONS	% POSITIVE
E. coli	15	9	60
Proteus species	14	14	100
Klebsiella-aerogenes group	18	13	72
Ps. aeruginosa	4	4	100
Alcaligenes fecalis	2	1	50
Staphylococcus (coagulase-positive)	4	3	75
Staphylococcus (coagulase-negative)	2	2	100
Candida albicans	4	4	100
Total	63	50	79.4

Infected Urine Specimens.—Positive catalase tests were obtained with 79.4 per cent of the 63 urine specimens that contained more than 10,000 bacterial colonies per milliliter. Catalase was found in all 14 urine specimens that contained Proteus organisms, in 72 per cent that contained Klebsiella-aerogenes, and 60 per cent of those infected with *E. coli*. The results are summarized in Table II.

*Schleicher and Schuell Co., Keene, N. H.

Twelve urine specimens contained less than 10,000 bacteria per milliliter and 6 of these exhibited catalase activity. Five of the urine specimens with catalase activity were from patients with unquestioned pyelonephritis; the other was from a man suffering from carcinoma of the rectum and contained a moderate number of leukocytes. The data from the twelve patients with less than 10,000 bacterial colonies per milliliter of urine are summarized in Table III. It is of interest that the urine from the diabetic patient with only

TABLE III. CATALASE ACTIVITY OF URINE CONTAINING LESS THAN 10,000 BACTERIA PER MILLILITER

CLINICAL DIAGNOSIS	BACTERIAL SPECIES	NO. BACTERIAL COLONIES/ ML.	DISK FLOTATION TIME* (MIN.)	SEDIMENT
1. Diabetes	Staphylococcus	720	10	Moderate pyuria
2. Alcoholic cirrhosis	*E. coli*	225	0	0
3. Chronic pyelonephritis	*A. aerogenes*	1,100	3	Heavy pyuria
4. Diverticulitis	*C. albicans*	170	0	0
5. Chronic pyelonephritis	*K. pneumoniae*	500	30	Moderate R.B.C., occasional W.B.C.
6. Diabetes	*E. coli*	370	0	Occasional epithelial cells
7. Cholecystitis	*E. coli*	6	0	Occasional R.B.C.
8. Unexplained fever	*P. mirabilis*	461	0	0
9. Rheumatic heart disease	*K. pneumoniae*	600	0	Occasional R.B.C.
10. Chronic pyelonephritis	*A. aerogenes*	1,000	3	Heavy pyuria
11. Chronic pyelonephritis	*E. coli*	2,700	5	Heavy pyuria
12. Carcinoma of rectum	*K. pneumoniae*	2,900	2	Moderate pyuria

*0 = no flotation.

720 colonies per milliliter in the pour plate was found to have innumerable staphylococci in the smear of the urinary sediment and on the surface of a blood agar plate streaked with a loopful of urine. This suggested that, because

TABLE IV. CATALASE ACTIVITY OF STERILE URINE FROM 16 HOSPITALIZED PATIENTS WITHOUT HEMATURIA OR PYURIA AS MEASURED BY DISK FLOTATION TIME

DIAGNOSIS	DISK FLOTATION TIME (MIN.)	COMMENTS
Transfusion reaction	10	
Fissure in ano	45	
Polycystic kidney	20	
Appendectomy (postoperative)	10	
Chronic pyelonephritis; diabetes	30	> 100,000 *K. pneumoniae* in urine culture 18 days earlier
Nephrosclerosis	60	
Nephrosclerosis	20	
Pyelonephritis	20	
Nephrosclerosis	40	
Gastric resection (postoperative)	10	Fever, paralytic ileus
Infectious hepatitis	35	
Chronic pyelonephritis	15	
Renal calculus	4	
Renal tuberculosis	15	
Cholecystitis	10	
Chronic pyelonephritis	35	> 100,000 *E. coli* in urine culture 12 days earlier

of the natural tendency for staphylococci to form a clump and appear as one colony, the pour plate may give staphylococcal colony counts that are erroneously low.

Sterile Urine From Hospitalized Patients.—Catalase was found in 45 of the 161 sterile urine specimens. Pyuria or hematuria accounted for the catalase activity of 19 sterile urine specimens but catalase was found in 16 other urine specimens that contained no blood or leukocytes and appeared normal by ordinary tests. The clinical diagnosis and urinary catalase activity of the sixteen patients with otherwise normal urine are given in Table IV. Renal disease was known to be present in eleven of the sixteen patients.

DISCUSSION

Measurement of urinary catalase by disk flotation appears to afford a simple but reliable method for distinguishing between normal and abnormal urine specimens. Although the full usefulness of measuring urinary catalase remains to be explored, the present study indicates that it may be of value not only for detecting urinary tract disease that could be further identified by microscopic urinalysis and culture, but also for recognizing renal injury in patients whose urine is otherwise normal. The catalase test for renal injury thereby serves the same purpose as the transaminase and other enzyme tests used to measure injury to various organs. The extreme simplicity of catalase assay permits its application at the bedside or under other conditions where microscopic and cultural analyses are not readily available. Whereas simple addition of peroxide identifies grossly pathologic urine, the disk flotation technique is required for recognition of the more subtle forms of renal abnormality and for quantitating catalase activity.

Additional studies will be required to account for the negative catalase reactions obtained occasionally with urine specimens yielding more than 10,000 bacteria per milliliter on culture. Perhaps the period of observation for disk flotation would have to be extended beyond 60 minutes to detect such bacteriuria. It is also possible, however, that simple bacilluria may not produce renal injury and thereby fail to release renal catalase. Because net urinary catalase represents the sum of renal, exudative, and microbial catalase, traces from any of the three sources alone might not be discernable. A third explanation for negative catalase reactions is the presence of a catalase inhibitor. This was thought to account for the negative catalase reaction in a urine specimen loaded with staphylococci and obtained from a patient with thallium poisoning. Thallium was excreted in the patient's urine and believed to inhibit the staphylococcal catalase. It is also possible that drugs and metabolic products of certain illnesses may inhibit catalase and give false positive catalase reactions.

Only enterococci, among ordinary urinary pathogens, lack catalase and were not encountered in the present study. Enterococci have been responsible for only 4 per cent of urinary infections in this hospital,[5] but these infrequent infections should also give positive catalase tests of the urine because of the associated renal injury and pyuria.

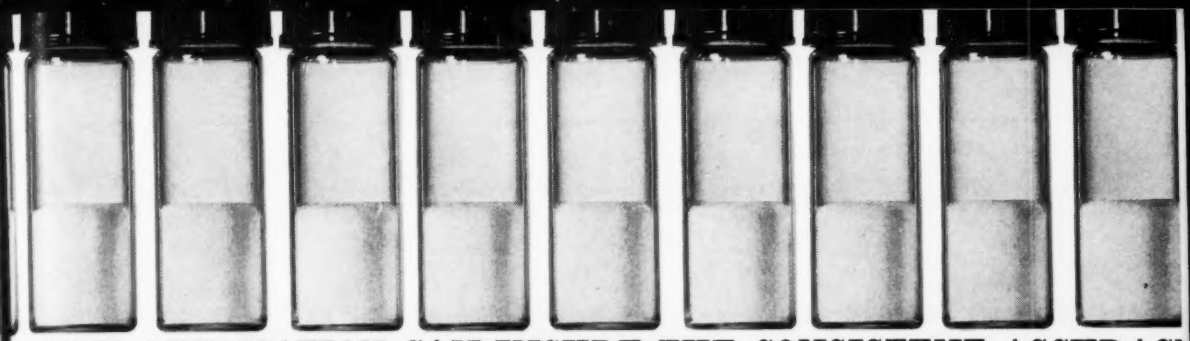
SUMMARY

Measurement of urinary catalase by the disk flotation technique of Gagnon and associates proved to be a simple method for distinguishing between normal and abnormal urine. Although catalase activity was not detected in normal urine, it was found in infected urine and in urine from patients with non-infectious renal disease. In a number of patients with renal disease, a positive disk flotation test was the only observable urinary abnormality.

We wish to express our thanks to Dr. Eugene Rice for his valuable advice and criticism.

REFERENCES

1. Braude, A. I.: Catalase Activity of Infected Urine, J. Clin. Invest. **38**: 990, 1959.
2. Lieberman, I., and Ove, P.: Enzyme Activity Levels in Mammalian Cell Cultures, J. Biol. Chem. **233**: 634, 1958.
3. Gagnon, M., Hunting, W. M., and Esselen, W. B.: New Method for Catalase Determination Anal. Chem. **31**: 144, 1959.
4. Sanford, J. D., Favour, C. P., and Mao, F. H.: Evaluation of the Positive Urine Culture Am. J. Med. **20**: 88, 1956.
5. Braude, A. I., and Siemienski, J.: Role of Bacterial Urease in Experimental Pyelonephritis, J. Bact. **80**: 171, 1960.
6. Jarvinen, K. A. J.: Determination of Peroxidase in Urine, Brit. M. J. **1**: 379, 1958.



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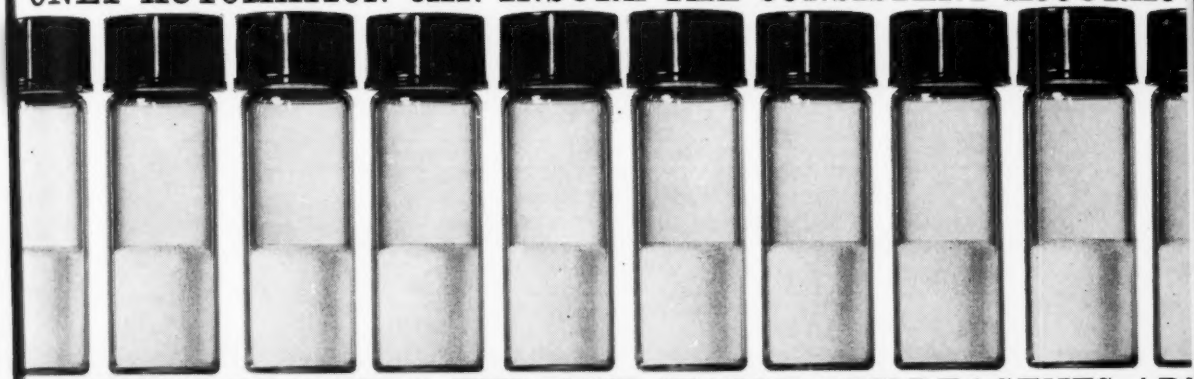

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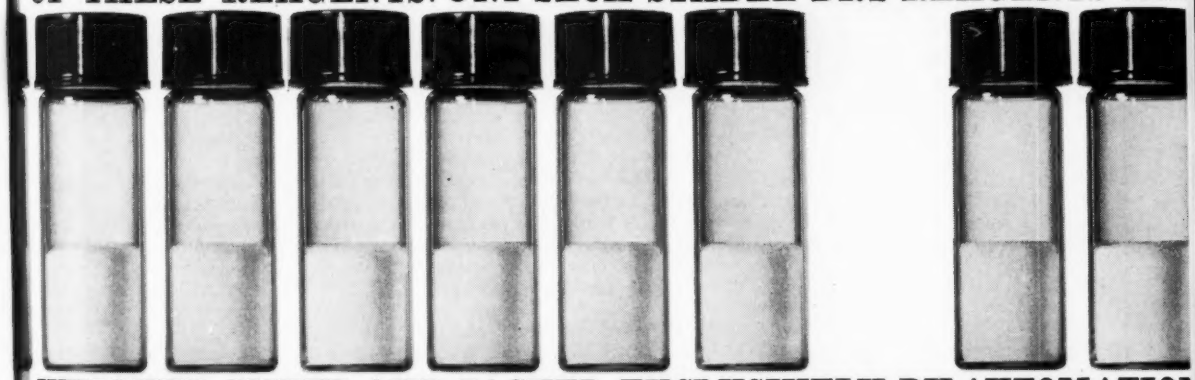

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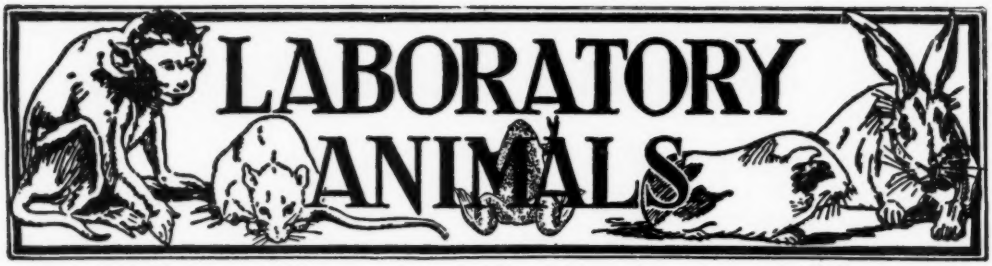

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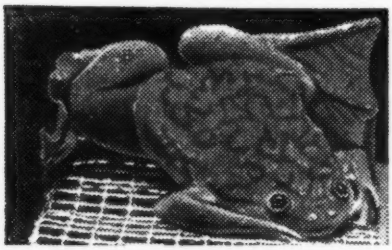

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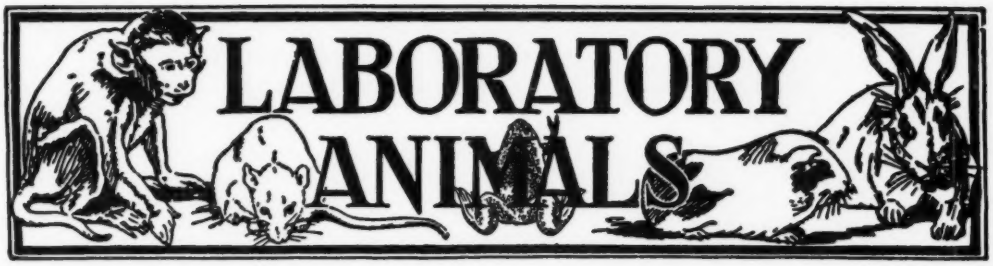

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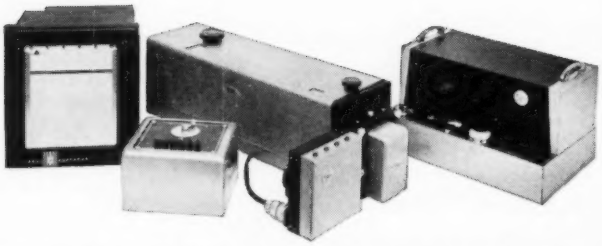

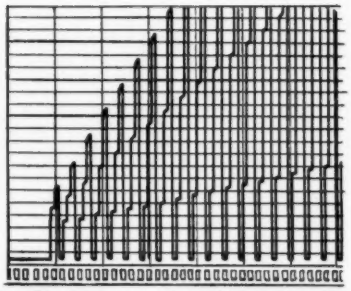

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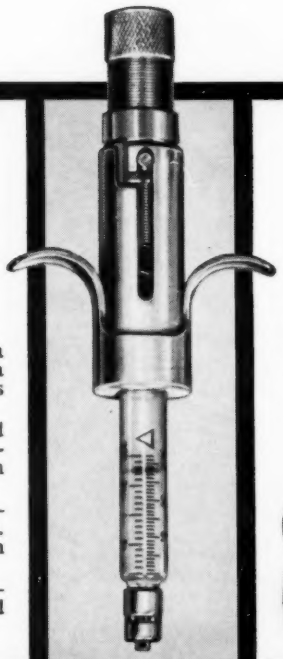

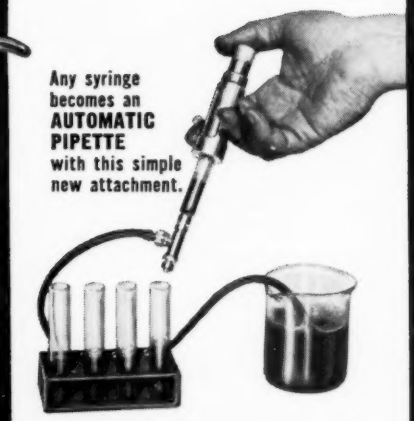

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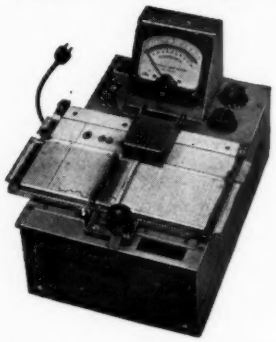

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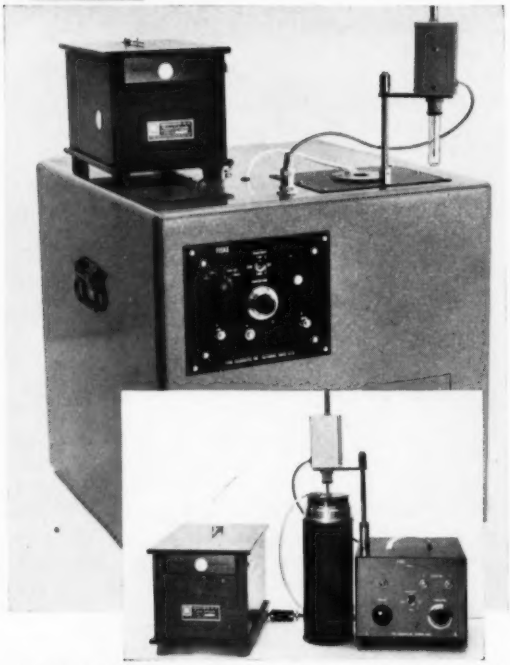

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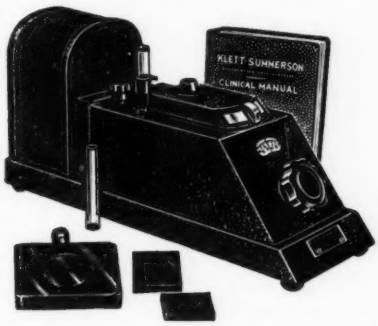

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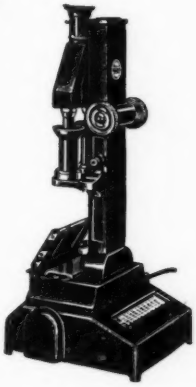

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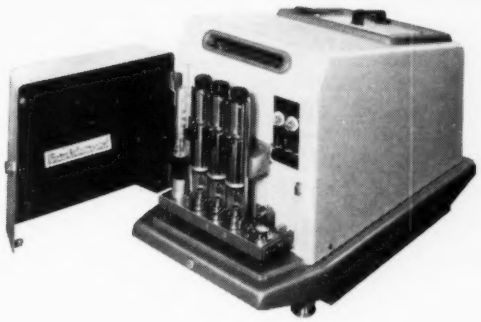

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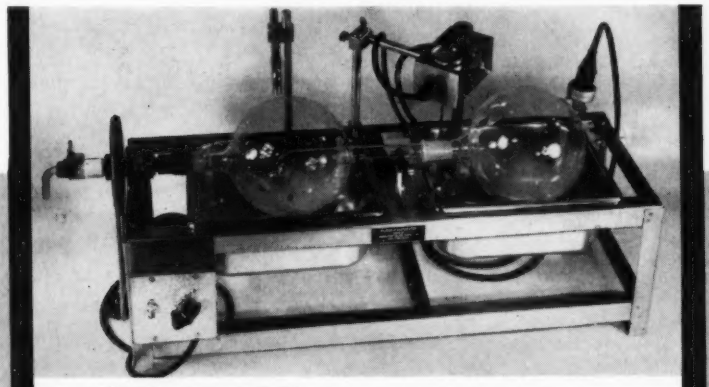

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is a dry powder requiring only the addition of ammonia and methyl alcohol to prepare the working solution for the staining of glycogen in paraffin sections and cytological smears. Dissolves readily. No Heating. No Filtering. No Ageing. Ready at once.

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One cc makes 50 cc working stain. Ideal for blood and bone marrow as well as malarial and blood parasites.

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PS1168	*Paragon Buffer Solution For Giemsa Stain pH 6.8*	*Bottle (100 cc)*	*1.00*
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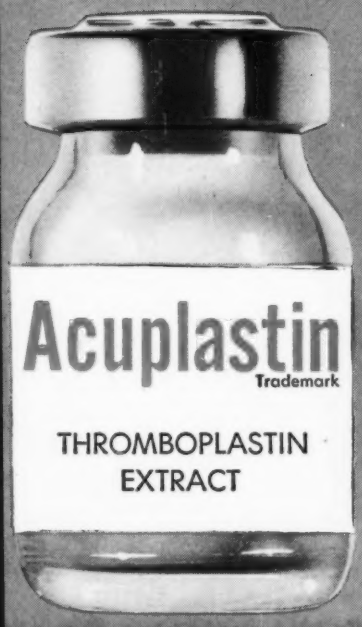

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